GENETICS

A PERIODICAL RECORD OF INVESTIGATIONS
BEARING ON HEREDITY AND VARIATION

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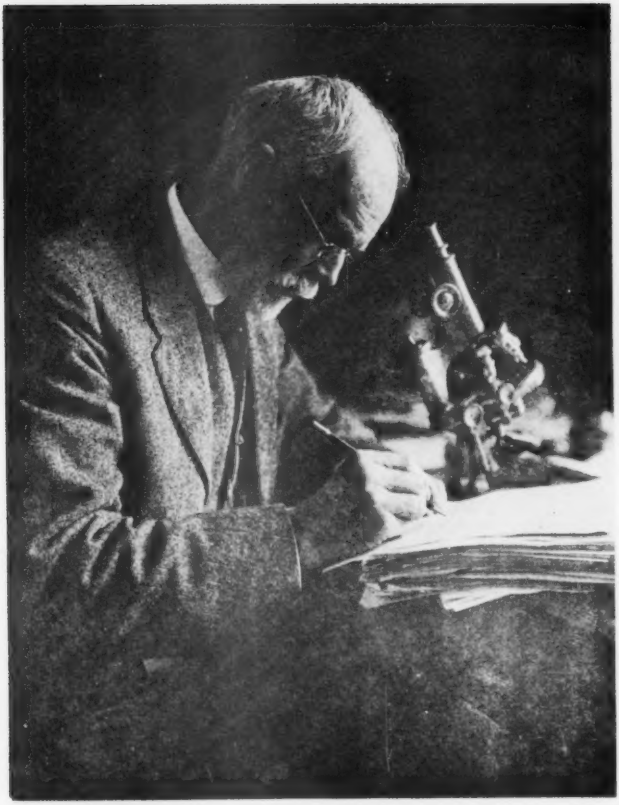

Edmund B. Wilson

E. B. WILSON

October 19, 1856—March 3, 1939

WHETHER we realize or not, we geneticists, as well as all general biologists who have acquired what we are pleased to regard as "the modern genetic point of view," see the phenomena of life through the eyes of WILSON. His was the balanced synthetic vision which, as much as half a century ago, sifted out and discarded the false and gratuitous, while collecting, conserving and fitting together the valuable and enduring elements of the then existing evolution, cell and chromosome theories of embryology, physiology and biochemistry, so as to form that consistent whole which we today accept as basic to all genetics and related fields. It is moreover surprising to look back from our present vantage point and see how much of our modern theory was already extant then, and how well the elements were joined so as to allow the incorporation of our later knowlecge without destruction of any essential parts of the structure as it then existed. As time went on WILSON continued to build up this edifice, both by constructive criticism of the work of others and by adding crucial building blocks of his own to it. Gradually, too, this system of concepts diffused throughout the world of biologists, through his writings, lectures and personal contacts, and through the confirming and extending effects of new discoveries, the origins of many of which could be traced in no small measure to his influence.

When in 1870–1871, at the age of 16, WILSON began seriously to study biology with a view to making it his life's work, the science as we know it did not exist. For mitosis and the chromosomes were virtually unknown, and evolution was an intruder, newly arrived upon the scene. In schools and small colleges practically no general biology was taught. During the ensuing decade however, WILSON had the good fortune to study at several institutions, under stimulating teachers in varied fields, —for example, at Yale, VERRILL in old-fashioned natural history and the stock breeder BREWER in heredity and evolution; at Hopkins, MARTIN in physiology and BROOKS in general biology. Abroad (1881–1882), in England and Germany, and at Naples, and a decade later again at Naples, WILSON came in contact with the most important researches and the most active minds of the day. He was possessed of a selective absorption for the best and the most significant that he came across, and he soon made the realm of theoretical biology, together with its most exacting new techniques, his own. In doing so he came to realize that, in consideration of the continuity of the germ plasm and the collateral position of the somatic tissues, that had been pionted out by WEISMANN, many of the chief clues for an understanding of ontogeny and heredity, and hence of evolution, must lie within the cell itself, particularly in the egg and sperm cells and in the processes of their formation during the maturation stages and of their transformation during early development. It is a brilliant illustration of man's continuing

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victory over nature that WILSON lived to see remarkable advances made in human knowledge in these very fields, advances in which, moreover, his own achievements played a cardinal role.

In WILSON's graduate work at Hopkins he had become started in descriptive cellular embryology, using Renilla, in which he showed that the same final result is attained despite striking variations in cleavage. In the decade 1881–1891 (during part of which he was at Bryn Mawr), he worked out the embryology of Lumbricus, where the same principle held to a lesser degree, and of Nereis, in which he proved the cell-lineage to have a remarkable constancy of cleavage pattern and of blastomere-organ correspondence. These two latter studies, considered by him in connection with one another and with those of other workers on widely different annelids, flatworms, molluscs and other types, not only gave clear evidence of the phylogenetic relationship of a great group of metazoa, characterized in their more primitive members by the teloblastic origin of their coelom and their precise spiral cleavage pattern, inherited from the distant past, but also showed that despite this prolonged persistence of ancestral ontogenetic features the fore-ordainment of embryonic cells was no absolute one, but subject to evolutionary and environmental modifications. For, as WILSON pointed out, some of the parts that are obviously homologous in the later stages of different forms are derived from non-corresponding cells of the early stages, even though these early stages themselves show patterns essentially identical with one another, as if the cells in question had been able to undergo a substitution of their embryological destinies. His experiments of a few years later in shunting nuclei of Nereis, by pressure, into regions of the blastula in which they would not normally be located, following soon after the similar work of DRIESCH on embryos of relatively "indeterminate" cleavage (sea urchins), showed that even in the most highly "determinate" forms there may still be an equivalence of potency of the early nuclei.

The decade 1891–1901, in which the above mentioned experiments were carried out, was that of the great furor in the field of experimental embryology that had just been opened up by ROUX, with his finding in 1888 that amphibian blastomeres, left alive after the killing of sister blastomeres, underwent, especially in their earlier stages, partial development. DRIESCH in 1891, on the contrary, found total development to ensue in isolated sea urchin blastomeres, and so the vehement controversy started in which ROUX, WEISMANN, and their followers claimed that the hereditary materials underwent an elaborate somatic segregation, all to their assigned places, goose step style, while DRIESCH stood out equally strongly for equivalence of blastomeres and for the determination of the development of parts by a continuous (and shall we say democratic) adjustment process, which, however, he interpreted ever more mysteriously until he ended with the postulate of "entelechy."

WILSON, again at Naples in 1892, took his place in the advance guard of experimentalists, joining this method to that of observation of "the experiments of nature," as he aptly termed the comparative method. He found in Amphioxus, in that year, cases of blastomere development even more toti-

potent (or "indeterminate," as CONKLIN called it) than in sea urchins, while CRAMPTON, as his student, soon found in the gastropod Ilyonassa, and WILSON himself later found in Cerebratulus, Dentalium and Patella, mosaic ("determinate") development more rigorous that in amphibia. Yet even in these extremes of each type he noted some traces of the other, and he gave reasons for the conclusion that both were manifestations of substantially the same series of processes, differing mainly in the time and finality with which the assignment of roles to the respective parts occurred, in relation to the time of the cleavage divisions. In all cases the early ovarian egg cytoplasm lacked "prelocalization" of embryonic parts. The prelocalization, occurring later, was in all cases "epigenetic," but in some kinds of eggs (for example, Dentalium and Patella) it occurred before maturation, in others (Cerebratulus) between maturation and fertilization, while in still others (Amphioxus, sea urchins) there was little if any prelocalization (at least within a given horizontal plane) until after some cleavages had taken place, and even that might not yet be final, but still subject to "regulation"—often as late as the adult, when as WILSON pointed out it constituted "regeneration"—through the interactions of one part upon another. The prelocalizations, whether early or late, and whether or not between cells or parts of cells (in fact, cell boundaries often seemed relatively unimportant, as WILSON pointed out in qualification of HERTWIG'S views), were in all cases expressions of such interactions, as a result of which the determination of the nature of a part was ultimately dependent upon its relation to the "field" that had been established, at what we should now call the "critical period," in the whole. The nature of these interactions, WILSON recognized, could receive its ultimate explanation only in terms of chemistry—a chemistry more advanced than then existing.

In reaching these interpretations, WILSON had the advantage, over most students of the embryology and heredity of that day, of possessing a physiological as well as a morphological training and viewpoint. He did not allow himself to be deceived by the facile artificial distinctions and imaginary constructions put forth by those of more purely morphological outlook, but thought of biological systems in terms of working, chemically interacting arrangements. In this textbook "General Biology" (1885, 1895), written with SEDGWICK, he performed the important service of introducing the subject of biology in this manner to the beginning student.

But long before the final chemical analysis could be attained, it was imperative for the biologist to gain what evidence he might concerning the derivations of the visible structures of the cell and their relative roles in cell functioning, development and heredity. As for the centrosomes, it was shown by WILSON in 1895, with his student A. P. MATHEWS, that those of the cleavage stages are derived from the sperm, as on BOVERI'S theory, even in the echinoderm eggs where FOL had pictured his peculiar "quadrille of centers" (erroneously involving both egg and sperm centrosomes). Making soon afterwards the first really critical cytological observations on artificial parthenogenesis (in 1901, directly after its discovery by LOEB), WILSON found, however, that the newly induced, normally functioning centrosomes, in this case,

could be formed from ominipresent cytoplasmic "ground substances" in the egg. As for the visible rays and fibres of mitosis, WILSON finally concluded—chiefly on the basis of his experiments involving anaesthetics, and amphiastral displacements—that they are artifacts, but that they are representative of lines of streaming or tractive force, organized from the centers (and perhaps involving an ultramicroscopic orientation of particles), and that these tend to cleave the cytoplasm and to pull the chromosomes, much as on BÜTSCHLI's theory. The preliminary metaphase separation of the chromatids, however, proved in his studies of monasters to be independent of this pull. And both chromosome and centrosome division were shown, through his studies on egg fragments, to be secondary to rhythmic changes initiated in the cytoplasmic ground substance. It was, moreover, in this ground substance, rather than in the visibly "formed structures" of the protoplasm, that the differentiations of prelocalization presumably occurred—a point to which WILSON added experimental evidence of his own many years later (1929), in his demonstration that egg fragments of Chaetopterus in which the formed substances had been removed or maldistributed by centrifuging nevertheless could develop practically normally. The basic role of the ground substance in cytoplasmic economy had been further emphasized, in WILSON's studies of the 90's, by observations on various eggs in which he showed the final alveolar structure to be derived by the enlargement of minute granules of the cytoplasm. This supported the view according to which neither a visibly reticular, alveolar or fibrillar structure of cytoplasm is primary. And it again left the problem of cytoplasmic organization confronting the frontiers of chemistry.

However this might be, it was borne in on WILSON more and more that the organization of the egg cytoplasm was itself an "epigenetic" one, fashioned under the control of the nuclear and, more specifically, of the chromosomal material. In 1884–1885 four men—O. HERTWIG, STRASBURGER, KÖLLIKER and WEISMANN—had independently come to the conclusion that this chromosomal material ("chromatin") "contains the physical basis of inheritance." This theme was taken up by WILSON and developed in a series of semipublic lectures in his first year at COLUMBIA, in 1892–1893, and further elaborated in his great book, "The Cell in Development and Inheritance," the first edition of which appeared in 1896 and the second in 1900. Here (already in 1896) he presented in ordered array the great mass of significant data which had by that time accumulated on the structure and functioning of the cell in general, and more especially in its processes concerned with reproduction, and, as the leitmotiv of his book, he showed how all the main facts pointed in the direction of the above cytogenetic conclusion. The most telling of these facts he listed under the following four heads: (1) the evidence furnished by observations on the preferential proximity of the nucleus to more actively anabolizing parts of cells, and by experiments showing that enucleated, unlike nucleated, cell fragments are unable to continue their reproductive or developmental functions, or even their other activities involving constructive metabolism; (2) the complicated series of changes which result in the constellation of chromosomes provided by the sperm and egg being almost exactly equivalent to one another,

in correspondence with the already known equivalence of father and mother in heredity, and in contrast with the extraordinary differences between sperm and egg in respect to their other parts; (3) the evidence (RABL, BOVERI, HÄCKER, etc.) that despite the obscurement of the chromosomes in the resting stages they retain their individuality—or, as WILSON called it, their "genetic continuity": this was based on their reappearance in similar positions and with the same numbers and morphological types as those which they had had previously, even when these original configurations had for any reason been abnormal; (4) the otherwise unintelligible precision with which each chromosome became divided, and indeed divided in all its parts—namely longitudinally,—a fact which, as ROUX in 1883 had pointed out, argued not merely for each chromosome but each part of each chromosome, in linear arrangement, being of distinctive character and important for succeeding cell generations. Even in 1896, however, WILSON agreed with STRASBURGER that the "ultimate dividing units" of the chromosomes were probably smaller than the visible chromomeres; furthermore, as he then stated, "these units must be capable of assimilation, growth, and division without loss of their specific character."

These units of assimilation, or, as we should now say, "genes," were thought of by WILSON, following CLAUDE BERNARD and KOSSEL, as controlling the character of the cell by determining the character of its synthetic processes, and he leaned here also, in 1896 et seq., to DRIESCH's suggestion that they worked through the production of distinctive enzymes. For the further explanation of how these synthetic processes were brought about, he looked to their peculiar composition as compounds of nucleic acid and protein as holding an eventual key. Thus their role as the physical basis of heredity was to be referred to their chemical action in causing the "recurrence, in successive generations, of like forms of metabolism," and the peculiar "cytoplasmic field" found in egg and embryo was "created and moulded" by these chromosomal elements.

For producing such effects it was not necessary for the hereditary elements to undergo a somatic segregation, as postulated by ROUX and WEISMANN. Since cytological observations were opposed to this and both pressure experiments on nuclei and regeneration experiments had in fact given evidence of the substantial equivalence of many diversely destined somatic nuclei, WILSON rejected the somatic segregation scheme entirely, together with its formalistic representation of individual parts or characters of the organism by single chromatin elements. He was too familiar with the complicated interactions occurring in actual embryology and physiology to be caught by any such naïve morphological views. Along with these he also rejected WEISMANN's fantastic grouping of the hereditary elements into an imaginary hierarchy of "biophores," "determinants," "ids" and "idants." On the other hand, he recognized the value of WEISMANN's arguments for the existence of some sort of qualitative reduction division. Despite the confused state in which the cytology of reduction was at that time, he pointed out that the very peculiarities and intricacies of these processes, ending as they were known to do in a reduced number

of chromosomes, held promise of offering important clues to the whole picture. And he himself with successive students plunged energetically into the attack on the problems of the synaptic period.

With this magnificient integration of all that was best in cytology, it was not surprising that, so shortly afterwards, when Mendelism had been rediscovered, the study of its material basis underwent such rapid development, nor that the lead in this development soon came to lie in the United States, a country hitherto little known for its biology, nor that, within the United States itself, the developmental gradient appeared to have its center of concentration in the neighborhood of WILSON. It was no accident that it was one of the students whom WILSON had set to working on synapsis, SUTTON, who, obtaining critical evidence for the truth of MONTGOMERY'S inference of the existence of homologous chromosomes, at the same time (1902) showed that these homologues in their segregation provide the material basis for the operation of both of Mendel's laws of hereditary transmission. Nor that, after MCCLUNG (who had shortly before got his start in cytology under WILSON'S guidance) had deduced the probable function of the X chromosome as sex determiner, it was WILSON himself, as well as STEVENS, who demonstrated the principle fully and unravelled its details, thus providing the first proved case of a known character—and one of first rate importance—whose determination must be attributed to a given chromosome. Then followed WILSON'S working out of further important ramifications of the theory of sex determination: the variations in X's and Y's, the degeneration of the Y and its relative inactivity, the dependence of sex upon the amount of specific X-borne material interacting with the rest of the chromatin, the compoundness of inner constitution of the sex chromosomes, their occasional non-disjunction leading to XO forms, etc. Moreover, all this, as well as correlative studies, gave unequivocal support to the doctrine of chromosomal individuality, and that of chromosomal compoundness, indicative of a genetic individuality of the still finer parts, and so, too, it led directly to his deduction of rearrangements of these parts having occurred in evolution. But while it was to be inferred that the "ultimate dividing units" were the Mendelian factors, it was, as WILSON insisted even in 1911, quite fallacious to postulate that they stood for any absolute "unit characters," since characters must be produced by complex interactions between the products of many genetic units, as well as environmental factors. Examining next the stages of synapsis, WILSON found the case clear cut for that longitudinal pairing of homologous threads which was a necessary condition if the chromosomes were to undergo some sort of orderly interchange of these finer parts, as the vehicles of many separable Mendelian factors would have to do. All this material was published in WILSON'S classical series of "Studies on Chromosomes" (1905–12), which marked the highest peak of his researches.

The thrill of these new discoveries inspired even the undergraduates studying biology at Columbia College, and a crop of them, growing up under the influence of the courses given there by WILSON himself, as well as of those given by WILSON'S former students, were attracted into the Drosophila work

which MORGAN had found to provide so fruitful a lead for the investigation of these questions. WILSON encouraged them in this and appeared as a chief advocate of their results before biologists elsewhere, and the cytological and genetic arms thenceforth advanced in ever closer union.

Although WILSON's health was never good after 1920, he remained actively at work in the preparation of his stupendous third edition of the "Cell" (1925). This volume in its comprehensive sweep succeeded in coördinating the bewilderingly ramified results that had accumulated in the hands of the triumphantly expanded science of cytology. But although, in 1927, this book received from the learned societies the official recognition that was certainly its due, it is a reflection on the conduct of such organizations that these honors had been merited at least equally by the brilliant pioneer work of some thirty years before, which had pointed straight forward in the direction of just those conclusions which the intervening years saw generally accepted.

Despite all this vindication of cytology, it was evident that, as WILSON observed in 1920, "for the time being genetic development of the chromosome theory has far outrun the cytological." Thus, partly as a result of its very victories, cytology had now, for some problems, become an alternative instrument. And although WILSON believed that after a while cytology would "close the gap"—as has in fact occurred since then—he preferred in the meantime to conduct his own researches chiefly along those lines in which cytology remained the only competent method. Some of this work of his later years upon the organization of the egg has already been referred to. In addition to this, WILSON and his coworkers during this period carried through a series of valuable researches on the achromatic formed bodies of the cell: especially the Golgi bodies, neutral red bodies, and chondriosomes. There had not hitherto been observations on these elements of comparably critical quality to those which, in the case of the chromosomes, had resulted in the elucidation of the role of the latter in heredity, and such cytological studies on the cytoplasmic bodies were especially needed in view of the lack of evidence about them from the genetic side and the claims that had been made for them in some quarters. The upshot of the work of WILSON and his colleagues on these bodies was to show that they do not in fact become divided and transmitted with the precision characteristic of the chromosomes and of known hereditary factors: they could, in consequence, not be well fitted to serve as vehicles for the transmission of distinctive characteristics through an indefinite series of generations. Thus in the end this evidence, too, redounded "to credit of the chromosome theory of heredity," by giving evidence of the uniqueness of the chromosomal role.

In WILSON's antecedents and early environment we may find a part of the basis for his character and achievements. Among these were the spirit of craftsmanship together with the love of adventure of his sea-going forefathers; the high degree of intellectualization which this tradition had undergone while his ancestors were in New England, and its attainment of a superlative expression in art (music); this had not inhibited the further development of resourcefulness, independence of judgment, and democratic comradeliness as

the family settled in what was then "the West" (Illinois). But had this sufficed to make him what he was there would have been hundreds of E. B. WILSONS. Rare and invaluable indeed was his combination of balancing character traits: of inner fire with calm controlled objectivity; of deep artistic feeling—seen in purest form in his exceptional ability as a musician, but also evident through all his works—with his keen scientific interests; of passionate attachment to the individual minute objects of his exquisitely painstaking labors with a drive toward their organization into those grand symphonies which brought order to the whole; of historical perspective and scholarliness with creative drive on his own part; of respect for the work of predecessors and rivals and assiduousness in culling from them every iota of truth with a bold readiness to reject everything which his unbiased and critical judgment could not support. In him was to be found conservation in that higher sense in which it exists but for the service of progress. For all creation is selection and recombination of preëxisting elements of one kind or another, sometimes more, sometimes less obvious. It is true, from this point of view, that even the sculptor but uncovers in the stone the statue there implicit which only he could discern. This very instance serves, however, to disclose the truly creative character of such "selection."

In countries and in fields of science lying farthest out of the range of WILSON'S earlier works, the conquests of the chromosome theory of heredity that became so widely known in the years 1915–1920 came at that time with a startling suddenness, and many were under the illusion that this set of conceptions had just been born: some accepted, while others rejected it, equally violently. True, there was a vague recollection of the doctrine of "Weismannism," but that was supposed to have long been disproved, and the present chromosome theory was considered to be a "neo-Mendelian" upstart. These observers from afar were not aware that all the stages had stood in direct legitimate descent from one another, and that already in the first decade after the suggestions of WEISMANN, ROUX and the rest, synthetic forces had got to work, shaping from these and from the other knowledge of the day a better balanced body of biological thought and more effective methods which, joining without a turn into the broadening path of the Mendelians, had led without a break, nor loss of any of the essential concepts, to our present day viewpoint in genetics. In America, however, many of us are well aware of this, for the influence of WILSON was both wide and deep.

One important reason for the extent of WILSON'S influence is because, besides his qualities as a scientist, he had those human attributes which led to the spreading of his personality. Kindly, scrupulous and regardful of others, he won the affection of his students and colleagues and, both in his lectures and personal contacts, he gained their utmost coöperation. He realized, moreover, that in science, as in art, ideas are not socially useful unless mediated through adequate communication. His every course, every lecture and demonstration was a model of perfection: in factual content, in organization, and in style. Similarly, his personal life expressed the beauty of his nature. Genet-

icists may well feel proud that they trace their intellectual and emotional lineage so largely through WILSON, and it will continue to be a help to them in their progress in all the days to come that they have learned to see their subject through his eyes.

H. J. MULLER

This biography was received in 1942, but delayed in publication by external circumstances.

The accompanying portrait of E. B. WILSON was taken in 1920 by Dr. A. F. HUETTNER.

HEXAPTERA, A HOMOEOTIC MUTANT IN DROSOPHILA MELANOGASTER

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ACCORDING to BATESON the term "homoeosis" refers to alterations of an organ of a segmental series from its own characteristic form to that of some homologous organ of the series. Several homoeotic mutants have been discovered in *Drosophilia melanogaster*, which result in replacement of certain organs of the normal fly by parts resembling other organs. Thus, arista is changed into a leg in aristapedia (BALKASHINA 1929), and into wing-like structures in a mutant mentioned by GOLDSCHMIDT (1945). Compound eyes bear palp-like structures in ophthalmopedia (GORDON 1936), kidney (VALADARES 1937) and its alleles, and in erupt (GLASS 1944). Mouth parts are changed into leg-like organs or into aristae by proboscipedia (BRIDGES and DOBZHANSKY 1933). Halteres are changed into wings in bithorax (BRIDGES and MORGAN 1923) and its alleles, bithoraxoid (BRIDGES and MORGAN 1923), and tetraptera (ASTAUROFF 1929). Wings are changed to halteres, "palps", and legs by tetraltera (GOLDSCHMIDT 1940, VILLEE 1942) and podoptera (GOLDSCHMIDT 1945).

The present paper describes the morphological expressions and the genetic basis of another homoeotic mutant, Hexaptera. In Hexaptera wing-like, haltere-like and leg-like appendages develop on the dorsal part of the prothorax. The bearing of this mutant on the concepts of homoeosis and homology will be discussed.

THE ORIGIN OF HEXAPTERA

Hexaptera was found in October, 1947 among individuals of about a dozen F_2 cultures descended from a cross of an Oregon-R wild type male by a female from the Muller-5 stock. The Muller-5 X chromosome ($sc^{81}B$ In-S $w^a sc^8$) contains a crossover suppressor and is viable when homozygous. Fortunately, the Muller-5 stock bottle that supplied the P female was still available, and upon examining 667 flies one male with Hexaptera-like phenotype was discovered. The spontaneous origin and heritable nature of Hexaptera were thus established.

Several non-heritable homoeotic changes involving the dorsal prothoracic region have been reported previously (WADDINGTON 1942, GOLDSCHMIDT 1945, and VILLEE 1946a). These anomalous outgrowths have taken the form of "shoulder excrescences", "wings", "halteres" and "palps". Some of these modifications may be considered phenocopies of Hexaptera.

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MATERIAL AND METHODS

The characteristic phenotypic expression of Hexaptera is the growth of additional appendages on the prothorax. A typical expression may be seen in figure 1 in which the prothoracic outgrowths are bilateral and pigmented. Because melanin pigmentation often obscures the morphology of the structures produced, specimens were cleared in a ten percent solution of KOH and mounted in glycerine before detailed examination. All figures except 1 and 4 are of flies prepared in this manner. The drawings have been made at a magnification of 100× with the aid of the camera lucida.

THE NORMAL PROTHORACIC REGION

As in other homoeotic mutants, the reaction norm of Hexaptera overlaps that of the wild type. In view of this it is desirable to describe the normal structure of the prothoracic region in *Drosophila melanogaster* and some of the variations which occur. This will enable us to understand some of the changes produced by Hexaptera.

The structures with which we are concerned are best seen from an anterior view of a decapitated fly (figure 2; see also ZALOKAR 1947). The pronotum, the dorsal part of the prothorax, is composed of a median and two lateral sclerites and stretches as a narrow collar across the thorax. It is closely fused laterally with another part of the prothorax, the propleurum (episternum 1), which lies between the base of the prothoracic leg, the pronotum, and the humeral callus (prescutum 2). The latter, whose segmental origin is uncertain, is a lobe-like structure usually bearing two large bristles (setae). The anterior thoracic spiracle lies in a sclerotized area which represents the intersegmental membrane between prothorax and mesothorax.

These structures show certain variations in non-Hexaptera flies. The humeral bristles are commonly changed in certain bristle mutants; however, the lower humeral bristle is often bent in normal flies as well. The humeral callus occasionally protrudes slightly at its antero-ventral margin, and the spiracle, usually without melanin, is pigmented in a few cases. Although typically the region of the spiracle is level with its surroundings, it sometimes forms a lobe-like structure (figure 3).

THE GENETICS OF HEXAPTERA

The dominant nature of Hexaptera

In studying the genetics of a homoeotic mutant it is especially important to establish, as phenotypic criteria for the identification of the mutant, expressions that do not overlap those of the wild type individual. In this study, flies were classified as Hexaptera only when their phenotypes involved changes other than the shape of the humeral callus and the nature of its bristles or the bulging of the spiracle region and its pigmentation. Both sides of the prothorax of each fly were examined under a binocular microscope (24×) and slight changes were checked using a magnification of 48×.

The following experiments furnish evidence that Hexaptera is due to a dominant gene.

1. Phenotypically Hexaptera flies were crossed to flies from a *Cy/Pm*, ds^{33k}, *H/C Sb* stock. One Plum Hairless Hexaptera female was found among 1274 F_1 offspring.

2. Fifty-five single pair matings were made between the F_1 of the above experiment and flies from an Oregon-R wild type stock. Of these, eight cultures produced a total of 13 Hexaptera among 467 offspring. The stocks used above had been carefully examined and found to contain no Hexaptera. The data come from cultures kept at 25°C and only include counts made in an interval of 18 days from the time of mating.

The symbol for Hexaptera is *Hx*.

The chromosome of Hexaptera

Flies from the crosses in which Hexaptera was first found had their X chromosomes marked with mutant genes. The normal distribution of the sex chromosomes from generation to generation showed that no Y chromosome is present in Hexaptera females. Since Hexaptera is transmitted to offspring by both male and female parents the mutant is not located on the Y chromosome. Moreover, since mutant males produce both male and female Hexaptera offspring in outcrosses to wild type females, this gene cannot be located on the X chromosome.

The possibility that Hexaptera is located on chromosome 4 was tested in the following manner. Phenotypically Hexaptera flies were crossed to flies from the ey^2 stock and F_1 sibs were crossed to obtain an F_2 generation. The F_2 progeny were: 749 non-Hexaptera (eyeless and wild type); 26 wild type Hexaptera; 2 eyeless Hexaptera. Since crossing over in chromosome 4 is extremely rare, phenotypically eyeless Hexaptera flies are proof that even though both fourth chromosomes are replaced Hexaptera is produced. Therefore, Hexaptera is not located on chromosome 4.

The second series of crosses in the preceding section is useful in determining the chromosome location of the mutant. The parents of the eight F_1 crosses that produced Hexaptera offspring had the following phenotypes: the female parents were wild type; 3 male parents were Plum Stubble and 5 Curly Hairless. The Hexaptera offspring were: 2 females and 1 male that were wild type for both markers; 8 Hairless females and 2 Hairless males. These data show that Hexaptera and Hairless underwent recombination in the gametes of the male parents. There are two possible explanations for this fact. One is that Hexaptera is located on chromosome 2 and therefore recombines with the Hairless third chromosome. The other is that Hexaptera is located on chromosome 3, that it is a normal constituent of the Hairless chromosome in the *Cy/Pm*, ds^{33k}, *H/C Sb* stock, but that it is not detected phenotypically in this stock due to a suppressive action of both the Curly and Plum chromosomes.

These hypotheses were tested in the following manner. A series of crosses was made between Plum Hairless flies (offspring from crosses containing Hexaptera) and Oregon-R wild type flies. The progeny that emerged during the

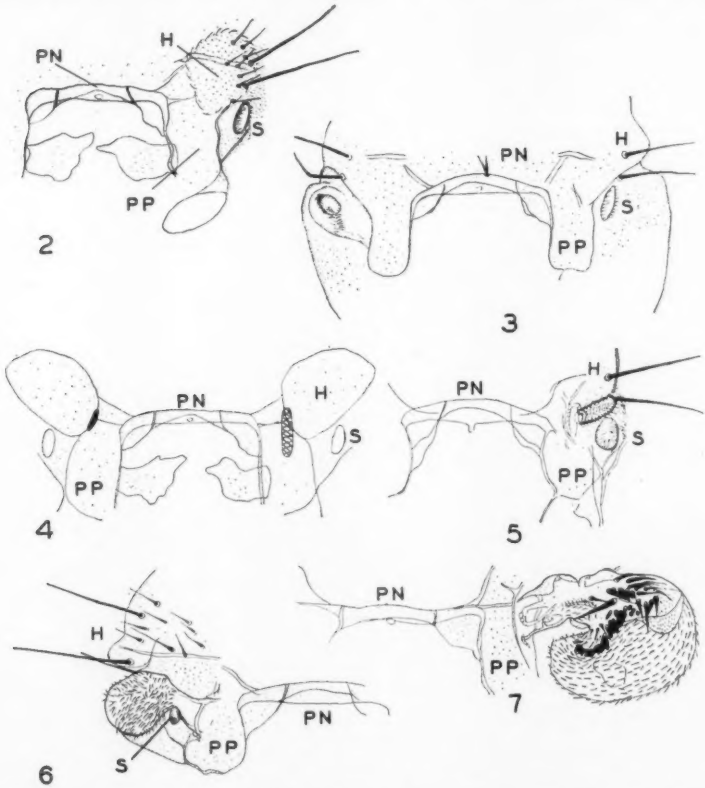
first 18 to 19 days from the time of mating were of the F_1 generation. The Hexaptera individuals in the F_1 were: 0 Plum; 11 Hairless; 0 Plum Hairless; 0 non-Plum non-Hairless. Using the same cultures, counts were continued after 19 days and though they undoubtedly contained some of the F_1 generation they primarily represent the F_2. The Hexaptera flies emerging after the 19th day were: 0 Plum; 21 Hairless; 14 Plum Hairless; 2 non-Plum non-Hairless. Note that there are Plum-Hairless Hexaptera flies in the "F_2" generation but none in the F_1. A 2×2 contingency table comparing the number of individuals that are Hairless Hexaptera and Plum Hairless Hexaptera in the F_1 and "F_2" generations yields a χ^2 value of 4.57. With one degree of freedom, this corresponds to a value of $P<.05$, showing that there is a statistical difference

FIG. 1.—Dorsal view of *Drosophila melanogaster* showing a typical expression of Hexaptera.

in the distribution of the Hexaptera individuals in the two generations and that the absence of Plum Hairless Hexaptera individuals in the F_1 cannot be due to small sample size. This fact is not consistent with the hypothesis that Hexaptera is a gene on chromosome 3. According to this hypothesis, if Hexaptera is lethal when homozygous, one would expect to find Plum Hairless Hexaptera individuals in the F_1 generation as well as in the F_2. If Hexaptera is viable when homozygous then this difference might be accounted for by postulating that the homozygote has statistically greater penetrance than the heterozygote. The data on page 17 may be utilized to calculate the effect of homozygosis on penetrance on the basis of the hypothesis that Hexaptera is on the third chromosome. Accordingly, when Plum Hairless flies (from crosses containing Hexaptera) are outcrossed to wild type flies one would expect 75 to 100 percent of the offspring to carry the Hexaptera gene; when these are outcrossed to Hexaptera flies one would expect 87 to 100 percent of the off-

spring to contain Hexaptera. If homozygotes have no greater penetrance than heterozygotes the ratio of Hexaptera flies in these series should be 75–100/87–100 or range from .75 to 1.15. If homozygotes show a greater penetrance than heterozygotes, since at least 3/8 of the offspring from outcrosses to Hexaptera flies are homozygotes, one would expect a significant drop in this ratio. The ratio experimentally obtained is .72. It may be concluded that these data do not show a detectable difference in penetrance between homozygotes and heterozygotes.

The double hypothesis that Hexaptera is on the third chromosome and is normally present in the balanced lethal stock is not in accord with fact.



FIGURES 2 to 7. Anterior views of decapitated flies about 50×. PN, pronotum; PP, propleurum; H, humeral callus; S, anterior thoracic spiracle.

FIG. 2. Normal prothoracic region.

FIG. 3. Non-Hexaptera variation in region of spiracle.

FIG. 4. Region of appendage attachment on dorsal prothorax; diagrammatic.

FIG. 5. Small wing-like Hexapteran appendage.

FIG. 6. Medium sized wing-like appendage.

FIG. 7. Highly differentiated wing.

On the other hand, all the data are completely consistent with the hypothesis that Hexaptera is located on chromosome 2. Upon recognizing that Plum and Curly chromosomes suppress the penetrance of Hexaptera and that Hairless enhances the penetrance of this mutant as much as or more than a wild type third chromosome, it can be seen that Hexaptera, as a second chromosome mutant, shows strict adherence to the laws of segregation and independent assortment. Therefore, it is concluded that Hexaptera is a dominant mutant located on chromosome 2.

Expressivity and penetrance in Hexaptera

The amount and kind of expression of Hexaptera is quite variable. Expression varies from the absence of a detectable difference fron normal flies, through various intermediate types, to the presence of a large appendage on the prothorax. Sometimes the entire structure remains beneath the exoskeleton and nothing at all is visible from the outside. The absence of external structure is due to the failure of the imaginal disc of the appendage to evert. These cases are usually detected in living flies by the presence of dark spots under the chitin of the anterior part of the thorax. At other times the entire structure lies above the surface. One also finds all degrees of outgrowth. However, the amount of outgrowth bears no relation to the total amount of structure produced.

Although it would be interesting to discover how the expressivity of Hexaptera responds to changes in genetic background and environmental conditions, such a study would be very laborious for several reasons. Not all flies that are Hexaptera are easily detected. Even with a magnification of 48× everted appendages may be so small that they remain unnoticed. Submerged structures that are not pigmented might often suffer the same fate. Finally, the morphology of the appendage is often masked by the pigment it contains.

The study of penetrance under varying experimental conditions is beset with fewer difficulties. It is necessary however to take precaution that the same criteria are used for identifying Hexaptera throughout the course of the experiments. In view of the relative rarity of normal variations in the prothoracic region, and since the penetrance experiments shall be interpreted relative to each other, it is adequate in this type of study to classify as Hexaptera all flies with changes in the prothoracic region.

The effect of residual genotype on penetrance

There are two simple methods for determining the effect of other genes on the penetrance of Hexaptera. One may use marked chromosomes that suppress crossover classes and note how penetrance is affected as one chromosome is substituted for another. Another way is to determine if sex produces a difference in penetrance.

The F_2 and F_3 generations from the original cross between an Oregon-R male and a Muller-5 Hexaptera female furnish data for analysis by both methods. When the Muller-5 X chromosome (marked by w^a and *B*) and the Oregon-R X chromosome (wild type) are both present in a female, the crossing

over between these chromosomes is suppressed almost entirely. However, while the integrity of these X chromosomes was retained in the F_2 and F_3 generations, the autosomes of the two stocks became similar.

In table 1 it can be seen that Hexaptera shows a statistically greater penetrance in females ($4.33 \pm .41\%$) than in males ($1.39 \pm .26\%$). In addition, as wild type X chromosomes are substituted for w^aB chromosomes, the penetrance in both males and females decreases. For example, the penetrance in $++/++$ females ($2.5 \pm .58\%$) is statistically smaller than in w^aB/w^aB females ($7.4 \pm 1.14\%$).

TABLE 1

The effect of residual genotype on penetrance. F_2 and F_3 progeny from the original cross between an Ore-O wild *type* ♂(++/*Y*) *and a Muller-5* (w^aB/w^aB) *Hexaptera* ♀.

GENERATION	GENOTYPE OF ♀♀	TOTAL ♀♀	*Hx* ♀♀	% *Hx* ♀♀	GENOTYPE OF ♂♂	TOTAL ♂♂	*Hx* ♂♂	% *Hx* ♂♂
F_2	w^aB/w^aB	388	32		w^aB/Y	120	1	
F_3		143	7			729	16	
Total		531	39	7.4 ±1.14		949	17	2.0 ± .45
F_2	$w^aB/++$	480	29					
F_3		820	25					
Total		1300	54	4.2 ± .56				
F_2	$++/++$	—			++/Y	568	9	
F_3		734	18			669	3	
Total		734	18	2.5 ± .58		1237	12	1.0 ± .28
Total		2565	111	4.33 ± .41		2086	29	1.39 ± .26

With the aid of similar methods, it was found that second chromosomes marked with Curly or Plum suppress the penetrance of Hexaptera more than wild type chromosomes, and that third chromosomes marked with Stubble show a weaker enhancing action than those marked with Hairless.

We may conclude that penetrance modifiers for Hexaptera are distributed throughout the chromosome complement.

The homozygote: its viability and effect on penetrance and expressivity

A homozygous stock of Hexaptera was produced by crossing a Plum/Hexaptera male by a Plum/Curly female and then mating sibs of the F_1. Since almost all non-Plum/non-Curly individuals in F_2 would be homozygous for Hexaptera, whether or not they showed the mutant character, such offspring were used to establish a homozygous stock. It may be noted here that Hexaptera flies seem hardy and fecund, and that the flies first emerging from crosses containing Hexaptera show a lower penetrance for the mutant than those hatching later.

Because establishment of a homozygous stock had to await the localization of Hexaptera on chromosome 2, and since the initial penetrance of this stock was low, data from other sources of Hexaptera are employed for determining the effect that the homozygote and heterozygote have on penetrance.

Two series of crosses were made with the F_1 from crosses between *Cy/Pm*, ds^{33k}, *H/C Sb* and phenotypically Hexaptera flies: Series 1 was 55 single pair matings to Oregon-R wild type flies, and Series 2 was 28 single pair matings to F_1 flies from crosses of phenotypic Hexaptera males by phenotypic Hexaptera virgin females. For both series, food conditions were the same and the temperature 25°C. Progeny counts were made for more than three weeks but since they cover similar intervals in both series of crosses the validity of the data will be unaffected. In the following, only crosses that produced Hexaptera offspring are considered. In Series 1, the Hexaptera gene is present in one-half of the gametes of the F_1 parent but is absent in the gametes of the other parent. Therefore, one-half of the offspring in this series will carry Hexaptera. In Series 2, the F_1 parents may carry Hexaptera in half their gametes or in none of them but is present in half or all the gametes of the other parent. In this series 5/8 to 8/8 of the offspring will carry Hexaptera. In Series 2 crosses there will be at least 25 percent more homozygous Hexaptera individuals in the progeny than in Series 1 crosses. The theoretical ratio for the frequency of Hexaptera in the offspring of these two series is: Series 1/Series 2 (4/8 divided by 5/8–8/8), that is, .5 to .8. One expects a lower value for this ratio since the residual genotype introduced in Series 2 crosses presumably favors penetrance more than the one contributed by the wild type flies in Series 1. Moreover, if the homozygote produces a greater penetrance than the heterozygote, one would expect a significant decrease in the value of this ratio.

In Series 1 there were 95 Hexaptera in 3839 offspring ($2.48 \pm .25\%$) in the 30 crosses that produced Hexaptera individuals; in Series 2 there were 95 Hexaptera in 2722 offspring ($3.48 \pm .35\%$) in the 22 crosses that produced Hexaptera offspring. The actual ratio of .025/.035 or .72 indicates that flies in which Hexaptera is homozygous have no detectably greater penetrance than flies that are heterozygous for the mutant.

It is highly desirable to determine whether or not homozygotes produce greater expressivity than heterozygotes. For this purpose, a semi-quantitative study was made by assigning numerical values to Hexapteran appendages according to size. Comparison between the Hexaptera flies in Series 1 and Series 2 shows no significant difference in the size of the appendages. This suggests that there is no difference in the expressivity in homozygotes and heterozygotes. Many mutant alleles, called dominant, actually do not produce dominant phenotypes since they have one effect when heterozygous and another when homozygous. From the description of the mutants of *Drosophila melanogaster* in BRIDGES and BREHME (1944) one finds less than a score of mutants that might be dominant in the strict sense. Hexaptera seems to be one of the few genes that are dominant in view of the fact that there is no detectable difference in penetrance or expressivity between heterozygote and homozygote.

The effect of population density on penetrance

In a cross that produces Hexaptera from 50 to 100 percent of the progeny will contain the Hexaptera gene depending on the genotypes of the parents. Therefore, when comparing penetrance in two crosses that produced Hexaptera in which the genotypes of the parents were undetermined one can only be wrong by a factor of two or so, since homozygotes do not produce more penetrance than heterozygotes. In view of the striking changes in penetrance wrought by overpopulation, our error in using data from crosses whose parental genotypes are unknown will not be significant.

Four types of culture conditions were employed in the experiments on the influence of population density. Type 1 cultures were those in which a normal amount of food was put into half pint bottles and in each one a single female parent was permitted to lay eggs for 2–3 days; Type 2 cultures were the same as Type 1 except that a normal amount of food (10 cc) was put into small containers called "creamers" (capacity 30 cc); Type 3 cultures were the same as Type 2 except that the female parent was not removed before her offspring emerged; Type 4 cultures were the same as Type 3 except that two females were placed in a creamer that had but half the normal amount of food. One or more males were supplied to each female and all cultures were kept at 25°C.

TABLE 2

The effect of population density on the penetrance of Hexaptera. See text for description of types of culture conditions.

CULTURE CONDITION	GENERATION	TOTAL ♀♀	% *Hx* ♀♀	TOTAL ♂♂	% *Hx* ♂♂
Type 1	F_4	569	0.9	459	0.0
Type 2	F_3	1284	3.0	1077	1.6
Type 3	F_3	868	7.0	678	1.5
Type 4	F_6	395	21.8	406	7.9

The data from these experiments are summarized in table 2 where it can be seen that as the degree of overpopulation increases, in progressing from a Type 1 to a Type 4 culture condition, the penetrance of Hexaptera increases correspondingly in both males and females.

Although there is a definite decline in penetrance in the three generations following the F_1, one cannot interpret the experimental results as due to a consistent selection of modifiers suppressing Hexaptera since in the F_6 generation the penetrance rises sharply.

The effect of temperature on penetrance

Ten females from stocks of Hexaptera with good penetrance were placed in a creamer, containing half the normal amount of food, with a similar number of males from the same cultures. Flies were permitted to mate and oviposit at room temperature for three days at the end of which the parents were trans-

ferred to fresh creamers. This procedure was repeated for each creamer every four to five days. The first creamers of different crosses were placed alternately in either a 20°C or a 25°C constant temperature room for the remainder of the flies' development. Creamers from any one cross were also alternated in this manner. Once hatching began, flies were removed daily when possible and counts were made so long as flies emerged.

TABLE 3

The effect of temperature on the penetrance in Hexaptera

	TOTAL ♀♀	% *Hx* ♀♀	TOTAL ♂♂	% *Hx* ♂♂
20°C	785	3.3	947	1.5
25°C	2066	24.2	2195	6.5

Table 3 summarizes the data from these experiments. The penetrance of Hexaptera in both males and females is greater at 25°C than at 20°C.

TABLE 4

The penetrance of Hexaptera on right and left sides of the body

	RIGHT SIDE	LEFT SIDE	TOTAL
Unilateral	344	326	670
Bilateral	348	348	348
Total	692	674	1018

Penetrance on right and left sides

Data on penetrance are useful in determining the nature of the relation between the production of Hexaptera on the right and on the left sides of the body. The results from six groups of experiments are presented in table 4. It is evident that Hexaptera is just as likely to involve the right as it is the left side of the prothorax. It may also be concluded that appendages are not produced independently on right and left sides, since the frequency of bilaterals in the total population examined is certainly greater than the product of the separate frequencies of the unilaterals.

THE MORPHOLOGY OF HEXAPTERA

Locus of attachment of the appendages

The region from which the Hexapteran appendages arise may be determined by examining the attachment of completely uneverted appendages, the splitting of sutures due to the presence of structures lying beneath the exoskeleton, and the basal connection of fully everted single appendages. In all cases the region of attachment is much the same and is shown as solid and cross-hatched areas in figure 4. The left attachment region (solid) was determined by the first two criteria and the right (cross-hatched) by the third; in all cases the attachment involved the suture between propleurum and humeral callus.

The shape of the basal connection of fully everted appendages varies from a slit to a circle. Most commonly it is an ellipse. When one accounts for the rotation which these appendages sometimes undergo, the morphology of the connection between appendage and body wall, regardless of the nature of the appendage, is basically the same (figures 6, 10, and 11). The posterior margin of the ellipse appears to be thicker than the anterior and has cord-like sclerotizations running from the appendage to the body wall both at its dorsal and ventral margins. The anterior margin of attachment is usually thin and merges gradually into the body of the propleurum. Though prothoracic appendages usually extend at right angles to the long axis of the body, only a part of the observed difference in sclerotization between anterior and posterior margins can be accounted for as an illusion of folding.

Other changes in the prothoracic region

Besides producing appendages, Hexaptera causes an increase in size of prothoracic sclerites. The lateral portion of the pronotum often grows toward the attachment region of an appendage while the dorsal propleurum becomes intimately associated with the anterior region of attachment (figure 11).

The morphology of the appendages

The fully everted appendages in figures 5, 6, 7, and 8 vary from a small structure barely recognizable as wing-like to a highly differentiated wing.

Although rather heavily sclerotized and shaped like part of a leg, the appendage in figure 5 is wing-like in nature. This is evident from its unsegmented condition and from the appearance of the hairs it carries which resemble those found on the membrane of the mesothoracic wing.

The appendage in figure 6 appears more like a wing than the one in figure 5 because it is less heavily sclerotized and has a better developed wing membrane.

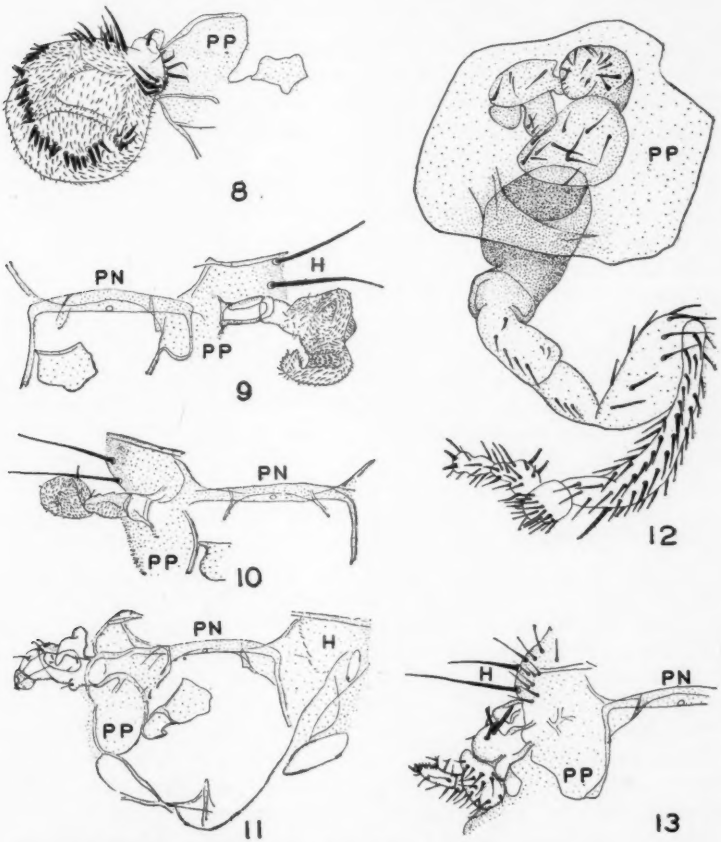
The wing in figure 7 shows the formation of a more complex structure for the attachment of the wing to the body as well as the development of venation. The double row of large bristles leading away from the wing base clearly represents the proximal part of the costal vein. The extra long bristle is probably a spur that corresponds to the single pair of spurs located on the normal wing just before the incision that separates the proximal and distal portions of the costal vein. Distal to the large bristles are short conical ones that resemble those found along the distal portion of the costal margin of the normal wing.

One of the most highly differentiated wing membranes found is shown in figure 8. It was a black balloon-like structure before clearing, and, in mounting, was slightly flattened to facilitate drawing. The bristle pattern is the same here as in figure 7 except that the venation of the wing margin is more developed. It is easy to distinguish the proximal costal vein of the anterior margin (double row of long thin bristles) from the rest of the marginal venation (short thick bristles). The surface of the membrane uppermost in the drawing, anterior on the fly, is considerably more developed than the underlying surface.

The appendage in figure 9 is unusual in that it is composed of two segments of which the proximal is leg-like and the distal clearly wing-like. Other flies

were found in which the appendage was composed of three segments. One of these is shown in figure 10. Here, the shape, the distribution of hairs, the presence of a few bristles on the distal segment, in addition to its trisegmented structure suggest a resemblance to a haltere.

In addition to wings, wing-like appendages, and halteres, Hexaptera produces legs on the prothorax (figures 11, 12, and 13). In many cases the leg had not everted completely although it was entirely above the body surface. This



FIGURES 8, 9, 10, 11, and 13. Anterior views of decapitated flies.

FIGURE 12. Dorsolateral view of left propleurum; all figures about 50×. Abbreviations same as for figures 2 to 7.

FIG. 8. Highly differentiated wing.

FIG. 9. Appendage with proximal segment leg-like and distal segment wing-like.

FIG. 10. Haltere-like appendage.

FIG. 11. Leg-like appendage, partially everted.

FIG. 12. Highly differentiated leg fully everted, leg beneath exoskeleton inverted.

FIG. 13. Everted leg with tarsal claw.

can be seen in figure 11 in which three segments are everted while three remain inverted. On the other hand, the leg in figure 13 is fully everted and one can recognize also a tarsal claw at the tip of the appendage.

The most highly differentiated leg found on the dorsal prothorax is shown in figure 12. Note in addition, the completely submerged and inverted leg rudiment. In the figure one can identify the following parts of the everted leg: three tarsal joints and claws, tibia, femur (twisted), trochanter, and coxa.

Multiple appendages on the prothorax

Although the majority of Hexaptera have single appendages there are a number of cases where several appendages occur on the same side. The fly in figure 12 has two extra legs on one side but no appendage on the other. Other flies have three extra legs or two extra legs and an extra wing. In those cases where a leg and a wing appear on the same side there does not seem to be any regularity in the relative position of the two organs. The locus of attachment for multiple appendages seems to be the same as it is for single ones.

DISCUSSION

Origins of the phenotypic effects in Hexaptera

The structures developed in the dorsal part of the prothorax of Hexaptera individuals are not found in normal flies. These structures may have several possible origins. Either an anlage, whose normal products form parts of the dorsal prothorax, is stimulated to increase in size, or other anlagen from the same segment or from different segments are stimulated to grow, split, consistently migrate and attach to a specific region in the dorsal prothorax. The latter possibility can be rejected since it requires a series of improbable assumptions and also because there is no evidence from studies of insect development for the regular migration of split or unsplit imaginal discs. On the other hand, the observations are consistent with the first hypothesis that a disc, normally present in the dorsal prothorax, is stimulated to produce tissue which develops into Hexapteran appendages. One may also account for those cases in which more than two supernumerary appendages are produced in one fly by assuming that in flies of this type a dorsal prothoracic anlage had split without subsequent migration of the split products.

How may one account for the great diversity in the type of organ produced from the differentiation of this imaginal tissue in Hexaptera flies? Some clues may be obtained from a consideration of the action of homoeotic mutants previously described in the literature. Either homoeotic mutants cause a shift in the time that a group of cells, normally produced, becomes competent to produce an appendage, or they change the time that different appendage evocators are released, or they do both. According to GOLDSCHMIDT (1940), it seems likely that homoeotic mutants affect the rate of differentiation of anlagen rather than change the nature of the evocators or the time of their release. This interpretation has been supported by VILLEE (1944, 1945, and 1946b) in his studies of the interaction of temperature and of "growth rate" genes with homoeotic mutants. As a consequence of the shift in the time that

anlagen become competent, they are determined by evocators that are different from the ones to which they usually respond, subsequently differentiating as organs or parts of organs that are not usually formed. GOLDSCHMIDT (1940) hypothesized that undetermined discs are totipotent in that they can become any type of appendage provided they are exposed to the proper evocator.

A simple working hypothesis is proposed here that adequately explains the diversity of the structures produced in Hexaptera and that is also in agreement with the interpretation presented above for the action of other homoeotic mutants. At a sufficiently early stage in development the prothoracic tissue would be still undetermined. This group of cells would have a wide prospective potency and during development evocators would be released which determine the fate of this tissue. Whether this tissue differentiated into leg, haltere, wing, arista, or mouth part would depend upon the stage in development during which these cells were produced, their prospective potency, and the kind of evocators that were active at this time. A possible complication arises because some flies have a leg and a wing on one side and a leg on the other side of the body. Such phenotypes might be due to the fact that although a disc splits it is not necessary that all the tissue in one of the split products becomes competent to react to evocators at precisely the same time as the cells in the other split product; consequently, any difference in their time of formation might lead to a difference in the kind or quantity of response that different cell groups manifest. Similarly, segmented appendages that are leg in one segment and wing in the adjacent one (see figure 9, for example) may be the result of different rates with which groups of cells within a disc become competent. Rather than involving a change in the time that the evocators for wing, haltere, and leg are released, it seems simpler to postulate that differentiations in the prothoracic region may be initiated by Hexaptera at different times in development subject to the action of the environment and residual genotype.

The bearing of Hexaptera on the concepts of homoeosis and homology

If the hypothesis of GOLDSCHMIDT regarding the mode of action of homoeotic mutants is correct, then these mutants do not cause a continuation of the ontogenetic development of an organ, but rather alter the entire course of development of undifferentiated imaginal discs. Once homoeotic mutants have opened new paths of development in this manner, the residual genotype can produce an organ or part of an organ that is different from the one usually produced in the body region concerned. No new organs have evolved subsequent to these single homoeotic gene changes; the structures they produce have evolved sometime in the past. A good example of this is found in Hexaptera. This mutant results in the development of a prothoracic disc that can differentiate into wing-like, haltere-like, and leg-like organs that are clearly the same types of organs as those formed elsewhere in the body. Presumably, the residual genotype that produces an organ in the normal fly is the same one that produces this organ when it appears on the prothorax. Since the homoeotic mutants thus far discovered do not seem to produce adaptive phenotypes these, like other non-adaptive genes, would be expected to have little, if any,

importance in subsequent evolution unless environmental conditions and the residual genotype undergo changes that make them selectively advantageous.

It is possible that in shifting development from its normal course homoeotic mutants can reveal an ancestral path that might furnish information concerning the past evolutionary history of structures. From a study of the phenotypes produced by the mutant podoptera, GOLDSCHMIDT (1945) has postulated that the evolutionary ancestor of both wings and legs was a "subdivided parapodium". One must agree with GOLDSCHMIDT when he points out that the specialized appendages in insects have probably arisen from simpler types of organs.

Homoeotic mutants frequently have been used to establish homology between different types of organs now in existence. Following HUBBS (1944) and ETKIN and LIVINGSTON (1948) the term "homology" may be used to describe a condition of similarity between two characteristics in organisms (structures, functions, behavior patterns, etc.) which results from their having had a dependent, rather than an independent, evolution. But the known cases of homoeosis in Drosophila suggest that the appendages of the adult can be formed from any undifferentiated disc. Studies of homoeotic mutants indicate that certain anlagen possess the potentialities to produce certain organs, but they do not prove that homology exists between the organs into which these anlagen differentiate. If anlagen that normally produce certain appendages are changed by homoeotic mutants they may differentiate into other appendages after exposure to appropriate evocators. For example, in certain homoeotic mutants eye anlagen are changed so that they form eyes bearing "palps", whereas in other mutants the anlagen of antennae are changed so that they form antennae which bear leg-like and wing-like parts. If only the evidence from homoeosis was considered in these cases, one might conclude that homology exists between these organs. However, morphologists (see FERRIS 1943) are by no means convinced that eyes and antennae are homologous to legs or wings

Nevertheless homoeotic mutants may serve to indicate homologies not previously established. Hexaptera is such a mutant. Insects in the Upper Carboniferous had wing-like appendages on their dorsal prothorax (see HANDLIRSCH 1919), but until the discovery of Hexaptera there had been no evidence of the presence of potentiality to form appendages in this region in present-day insects. Hexaptera throws some light on this problem, although it is unlikely that the wings of Carboniferous insects were the evolutionary predecessors of the wings, legs, and halteres in Hexaptera. A more reasonable supposition is that Hexaptera reveals a prothoracic anlage in *Drosophila melanogaster* that is probably homologous to the prothoracic disc in Carboniferous insects that produced wings.

SUMMARY

Hexaptera is a spontaneous, homozygous viable, dominant mutant located on chromosome 2 in *Drosophila melanogaster*. Its action results in the formation of appendages, frequently bilateral, on the dorsal part of the prothorax.

Hexaptera is a homoeotic mutant in the sense that the appendages produced may take different forms, such as wing, haltere, or leg. Penetrance is variable, dependent on the residual genotype, improving in densely populated cultures, and greater at 25°C than at 20°C.

A working hypothesis is proposed that Hexaptera causes a dorsal prothoracic anlage to grow and that the type of appendage which it forms may be determined in the same manner as it is in other homoeotic mutants. The bearing of this mutant on the concepts of homoeosis and homology is discussed.

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THE RELATION OF ATMOSPHERE TO BIOLOGICAL EFFECTS OF X-RAYS[1]

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FOR most of the past half century since the discovery of radium and X-rays, the effects of radiations from these sources upon living cells have been studied intensively. Although much information has been accumulated on the macroscopic and cytological changes produced, little is known of the mechanics of these changes or of the relation of environmental conditions to the effects of short-wave radiations. The present study is a contribution to the meager observations already recorded on the relation of atmosphere to the biological effects of X-radiation.

An effort was also made to determine the presence or absence of correlation (or cause and effect relationship) between injury symptoms, genetic effects, and chromosomal aberrations resulting from X-raying seeds (grains) of barley.

These experiments continue investigations begun by SMITH (1946 and unpublished).

LITERATURE REVIEW

Literature on the effects of X-rays on living cells has been summarized by DUGGAR (1936), GOODSPEED and UBER (1939), and LEA (1947), among others. Heavy doses of X-rays reduce viability, retard germination (both rate and percentage), stunt growth, and increase mutations and sterility. According to FRÖIER and GUSTAFSSON (1941) and GUSTAFSSON (1947), X-rays cause two types of cytological changes depending upon whether the nuclei are in interphase or actual division when irradiated. The primary effects, arising when irradiation is applied during prophase, are irregular fragmentation and an agglutination of the chromosomes resulting from "stickiness" of the matrix. As a consequence of this agglutination, the movements of the chromosomes are irregular and may lead to an atypical orientation of the spindle, degeneration of daughter nuclei, pseudo-amitoses, extension in time of contraction of chromosomes before metaphase, and other related aberrations. The secondary effect produced by irradiation of interphase nuclei, stems from aberrations resulting from fragmentation with or without subsequent rejoining of the fractured chromosome ends. It gives rise to translocations, inversions, deletions, bridges, fragments, and related irregularities. It is this second effect which causes genetic changes, but it has yet to be determined whether it is the first, the

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second, or some other effect which is primarily responsible for the physiological changes (injury symptoms).

Literature on the influence of atmosphere on X-ray effects is limited, but apparently there is less injury in the absence than in the presence of air. MOTTRAM, in 1935, reported that there was less inhibition in the growth of roots of *Vicia faba* if they were X-rayed in anaerobic rather than in aerobic conditions. The work of SMITH (1946 and unpublished) and THODAY and REED (1947) confirmed these results. SMITH mentioned preliminary data which indicated that there was less injury to dormant seeds of barley and einkorn when they were X-rayed in a vacuum or in an atmosphere of nitrogen than when they were X-rayed in air. This type of knowledge may also be of interest in X-ray therapy, as suggested by THODAY and REED (1947) who demonstrated that dividing cells were less injured if irradiated in the absence of air. Their results were considered to confirm observations of CRABTREE and CRAMER (1934) who had previously found "a similar effect of anaerobiosis on radiosensitivity of tumour cells." (Quoted from THODAY and REED 1947.)

One concept held by many authors is that damage from X-radiation is in general proportional to the dosage. TIMOFÉEFF-RESSOVSKY (1937, 1939) and others have felt that the number of genic changes induced by X-rays are directly proportional to the dosage and are independent of the wave length or time factor. BAUER (1939) stated that the number of rearranged or reunited contact points was proportional to the square of the X-ray dose. CATCHESIDE (1938) X-rayed *Drosophila melanogaster* males and noted that the number of structural changes induced in chromosomes showed a direct linear proportionality to the dosage between 1,000 r and 4,000 r. Later MULLER (1940), among others, concluded that the frequency of chromosomal aberrations increased somewhat more than the first power and less than the square of the dosage. SAX (1938) presented data showing that the frequency of chromosomal aberrations increased geometrically with X-ray dosage. These statements are indicative of the lack of agreement among authors as to the nature and relation of genic and structural effects induced by irradiation. Other works have been summarized in the discussion in this paper.

A second concept of concern to this report is the belief held by many workers that X-ray injury and lethality are due to genetic causes. FRÖIER and GUSTAFSSON (1941) stated that since chromosomes are the only parts of the cell with continued independent existence, any chromosomal disturbance would influence cell division and hence cell reproduction and related phenomena such as growth. They stated further that profound nuclear and chromosomal alterations are irreversible, whereas induced cytoplasmic changes can probably be reversed. Therefore, they concluded that seed mortality following irradiation is "probably an expression of chromosomal and genic changes." LEA (1947, pp. 341–344) was more cautious. He reasoned that since X-radiation kills bacteria and large viruses in the same proportions and under the same conditions that produce gene mutations in *Drosophila*, lethal action in bacteria and viruses might be the result of lethal gene mutations. However, he only suggested that such could be the situation in higher organisms. There is no proof as yet for

such a hypothesis, but on the other hand, there is evidence that chromosome structural changes cause the death of rapidly dividing cancer cells in which degeneration sets in only at the next division following irradiation.

THODAY and REED (1947) cited GRAY (1942) as being one of several authors who have hypothesized that the lethal effect of ionizing radiations is a result of abnormalities resulting from the division of cells with aberrant chromosomes. If this were the case, a decrease in injury symptoms should be accompanied by a parallel decrease in the number of chromosomal aberrations. In subsequent experiments in which roots of *Vicia faba* were X-rayed in three different media (oxygen, air, and nitrogen), they found that roots irradiated in oxygen grew less, and those treated in nitrogen grew more, than those treated in air. Correspondingly, there were more chromosomal abnormalities (bridges and fragments) in those cells irradiated in pure oxygen than in those treated in nitrogen. Similar results were obtained when carbon dioxide, nitrous oxide, and hydrogen were substituted for nitrogen. This suggests that even though physiological injury is ordinarily correlated with chromosomal changes, there must be some physical factor present which influences the degree to which X-rays damage the cells. THODAY and REED'S experiments most strongly point to oxygen and MOTTRAM'S (1935) and SMITH'S (unpublished) findings further suggest this possibility.

Other workers have also indicated that there may be factors involved in injury resulting from irradiation, other than merely genetic changes—for example, a chemical, physiological, or physical effect. FRÖIER and GUSTAFSSON (1941), and KEMPTON and MAXWELL (1941), among others, mentioned that there is a delayed killing action following irradiation that would be somewhat difficult to account for on the basis of induced genic changes, which presumably are more or less instantaneous. However, some chromosomal aberrations might develop their full effects only after several cell generations. According to FRÖIER, GELIN, and GUSTAFSSON (1941), nuclei of *Avena sativa* will divide even when the chromosomes are so distorted that they have lost their characteristic rod-like shape. They suggested that the extra-chromosomal influence that initiates mitosis may be inhibited by extremely high doses of irradiation. FRÖIER and GUSTAFSSON (1941) noted that KAPLAN (1940) had reported that pollen-tube nuclei of *Antirrhinum* were inactivated by 100,000 r, whereas pollen tube growth was possible after a treatment of 300,000 r. They suggested that perhaps the effect of such heavy doses of irradiation is extra-nuclear and probably chemical. They refer to KAPLAN'S (1940) theories: that irradiation causes ionizations which may destroy compounds of low molecular weight and give rise to poisonous compounds, or, that the rays may destroy the "Steuerungszentren" which are large molecules responsible for catalyzing and regulating physiological processes.

Specifically, the physiological factor in X-ray injury may be the inactivation of auxin or growth substances, or possibly even enzymes. FRÖIER and GUSTAFSSON (1941) noted that wet pollen was less susceptible to X-ray injury than dry pollen, and suggested that this resistance resulted from the ability of hydrated spores to replace auxin destroyed or inactivated by irradiation. SKOOG (1935),

in an outstanding work on the effect of X-radiation on auxins, noted that auxin was inactivated or destroyed by X-radiation and that the rate of inactivation was directly influenced by the presence of oxygen and the amount of auxin present. He then suggested that destruction of auxin is a major cause in the immediate inhibition of growth by X-radiation. GOODSPEED and UBER (1939) cited CHOLODNY (1935) as also suggesting that dwarfing following X-radiation is probably caused by inactivation of auxin. POPP and MCILVAINE (1937) showed, in a study using various long energy wavelengths between 250 mμ and 450 mμ that the reduction in growth substances was correlated with a decrease in the wave length of the incident radiation. In his summary of the biological effects of radiations on living cells, LEA (1947 p. 37) brought out the possibility of the inactivation of vital enzymes by irradiation, pointing out that enzymes in the dry state are inactivated by X-radiation.

Thus, whether there is a necessary (cause and effect) relation between genetic aberrations and physiological injury resulting from X-radiation is a matter of debate. Usually physiological reaction and frequencies of genetic changes are correlated, but critical experiments capable of distinguishing whether the correlation is chance or necessary are not common. SAX (1942) concluded that while chromosomal alterations may be the chief cause of injury, the physiological response of tissues to X-rays may be important too. GELIN (1941), from his work with dormant and germinating seeds of barley, stated that "after X-raying with the same dosage, the reaction of the chromosomes is directly influenced by the physiological condition of the seeds."

MATERIAL AND METHODS

In all experiments reported herein, seeds of Himalaya barley were used. The seeds were soaked in water about eight hours, after which they continued germination on moist blotters in Petri dishes for about ten hours. The water and Petri dishes were kept at room temperature. Germinating seeds were used for two reasons: 1) at the time the experiments were begun the X-ray apparatus available was not satisfactory for giving dosages large enough for efficient use of dormant seeds; and 2) because a given dose of X-rays produces a much higher frequency of changes in germinating than in dormant seeds. SMITH (1946) cited several authors who have demonstrated that the effect from irradiation is greatly increased with increased moisture content of seeds. FRÖIER and GUSTAFSSON (1941) noted that with desiccation there is a stabilization of nuclei, whereas, conversely, hydration increases nuclear lability to radiation (GELIN 1941). GELIN reported that an increase of 5 percent in water content of seeds (from 10 percent to 15 percent) increased chromosomal aberrations twofold. He suggested that possibly water weakens the bonds between atoms by penetrating, or being incorporated into, the chromatinic protein molecules.

Comparisons between the effects of a given dose of X-rays on seeds in air and in a vacuum were made as follows: the same number of seeds were placed in two similar pyrex test-tubes (taken from the same commercial carton). The tubes were tested and found to absorb approximately 60 percent of the irradiation so that with treatments of 3,000 r, for example, the seeds actually received

about 1,200 r. Hereafter, however, the doses reported are those applied at the surface of the seeds. Seeds that were to be irradiated in air were treated simultaneously with those that were sealed in a vacuum in the second tube. The vacuum was created by a suction pump designed to reduce the pressure to 0.0001 mm Hg. Although the degree of vacuum was never measured, results depending on this factor were consistent. While in the vacuum (insured by a greased stopcock) the seeds were carried $\frac{1}{8}$ mile to the X-ray machine. Every experiment included a control sample of seeds.

In several instances seeds in a third test-tube were X-rayed simultaneously for one or the other of the following additional treatments: 1) seeds were X-rayed in air after being in a vacuum for a period of time equal to the time the lot of seeds were in the vacuum during irradiation; or 2) seeds were X-rayed and then exposed to a vacuum for a corresponding period of time.

An X-ray machine operated at about 230 KVP and 3 ma was used for all the tests. The seeds were $7\frac{1}{2}$ inches from the target of the tube and received 50 r to 65 r per minute. Varying dosages were given ranging from 400 r to 5,200 r.

After being irradiated, the seeds were planted immediately. Germination was measured by the number of seedlings that emerged, and growth by the estimated average height. These data were obtained by growing the seeds in loam (one flat per test run in the greenhouse). The height was estimated by placing a ruler beside the clumps of seedlings. In one case additional data on germination were obtained when the seeds were planted in the field for a mutation study. Heat from a 200-Watt light bulb was applied in a few of the earlier tests to hasten growth processes.

To determine the seedling mutation rate, plants from treated seeds were grown to maturity. In the fall, seeds from individual spikes of these plants were grown in the greenhouse and the mutation rate was determined following the method of STADLER (1930). The mutation rate was based on the number of observable mutants appearing among 3- to 4-inch seedlings during a growth period of from ten days to two weeks. In some cases more than one kind of mutant was present among the progeny of a single spike. In such an event each different type of mutant was counted as an independent mutation.

To obtain cytological data, seeds were germinated on moist blotters in Petri dishes. When the roots were $\frac{1}{8}$ inch or less in length, they were fixed in Carnoy's fluid, formula A, and stained with acetocarmine following the technic described by SMITH (1947). Such a smear technic has especial advantage for this kind of study because it is rapid and because individual cells are isolated more or less in a single plane. It also avoids a pitfall of sectioning technics—namely, artificial production of fragments.

Chromatinic bridges at late anaphase or early telophase were used as the index of frequency of chromosomal changes. Bridges were used as criteria because they can be seen with considerably greater certainty than fragments. However, fragments were tabulated, because, though not so accurate, their frequencies did give indications that supported the data obtained on the frequencies of bridges.

EXPERIMENTAL RESULTS

A. The Effect of Irradiation in Air and in a Vacuum on Germination and Growth

In the first test, samples of 50 seeds each were given the following treatments: 1) one lot was left as a control, 2) a second was subjected to a vacuum for one hour, 3) a third was X-rayed while surrounded by air, 4) a fourth was X-rayed while in a vacuum, and 5) a fifth was subjected to a vacuum for one hour and then X-rayed in air. In order to determine the relation between X-ray dosage and atmosphere, four different dosages of X-radiation were ap-

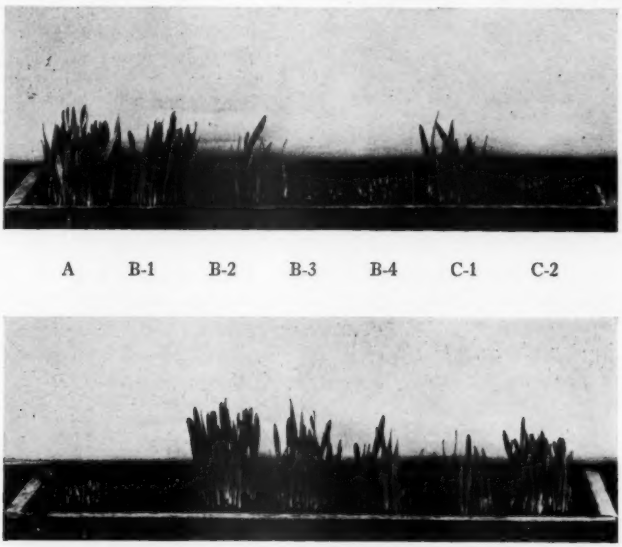

FIGURE 1.—A comparison of the effects of X-raying germinating barley seeds in air and in a vacuum as indicated by 14-day-old seedlings. A, Control. B, X-rayed in air: 1—400 r; 2—800 r; 3—1 200 r; 4—1,600 r. C, X-rayed in air after evacuation: 1—400 r; 2—800 r; 3—1,200 r; 4—1,600 r. D, X-rayed in a vacuum: 1—400 r; 2—800 r; 3—1,200 r; 4—1,600 r. E, Evacuated only.

plied to additional series: in one series the irradiation was 400 r, in the second 800 r, in the third 1,200 r, and in the fourth 1,600 r. The data from this experiment are presented in table 1. Figure 1 shows part of the plants from this same experiment. From this and other experiments, 1,200 r was chosen as a suitable dosage to show the contrast between irradiation in air and in a vacuum, and was used in most of the later tests.

Table 2 presents a summary of two succeeding tests. In addition to the treatments mentioned in test 1, a sixth sample of seeds was X-rayed in air and

TABLE 1

A comparison of the effects of X-raying germinating barley seeds in air and in a vacuum as measured by germination and growth.

X-RAY DOSAGE	GERMINATION (PERCENT)[1]					HEIGHT OF SEEDLINGS[2]				
	0	400 r	800 r	1,200 r	1,600 r	0	400 r	800 r	1,200 r	1,600 r
Treatment[3]										
Control	100					100				
Evacuated only	96					96				
X-rayed in air		100	100	92	94		96	31	11	4
X-rayed in a vacuum		96	100	96	88		100	88	81	62
X-rayed in air after evacuation		100	85	67	29		57	19	8	2

[1] Actual percentage of seeds germinated.

[2] After 14 days (percent of control).

[3] Applied to 50 seeds in each sample (a total of 700 seeds were involved in the experiment).

then subjected to a vacuum until the seeds had been without air for as long as those that were X-rayed in a vacuum had been.

The results indicated that in general those seeds which were X-rayed while in a vacuum had a higher germination percentage and in every case a better growth rate than those X-rayed in air. This trend was consistent in each of the 12 trials run to show effect on germination and growth, the data from three of which are presented in tables 1 and 2. In table 2 it can be noted that the height of seedlings from seeds X-rayed in air either before or after a period of time

TABLE 2

A further comparison of the effects of X-raying germinating barley seed in air and in a vacuum as measured by germination and growth. (Data from two tests.)

AGE OF SEEDLINGS	GERMINATION (PERCENT)[1]		HEIGHT OF SEEDLINGS[2]	
	Test A 11 days	Test B 12 days	Test A 11 days	Test B 12 days
Number of seeds in sample	50	25	50	25
Treatment				
Control	100	80	100	100
Evacuated only	98	—	111	—
X-rayed[3] in air	24	4	37	11
X-rayed in a vacuum	98	80	56	79
X-rayed in air after evacuation[4]	32	4	59	63
X-rayed in air before evacuation[4]	20	4	59	37

[1] Actual percentage of seedlings germinated.

[2] Percentage of control.

[3] 1,200 r units.

[4] Vacuum applied 35 minutes in test A and 60 minutes in test B.

in a vacuum was not reduced so much as those X-rayed in air without evacuation. However, the survival of seeds given the three treatments were comparable. In table 1 the relation between treatments and seedling heights are more nearly as expected. From table 1 and figure 2 it is evident that an inhibition of growth preceded a decrease in germination, that is, there was a much greater gap between growth of seeds given 400 r of X-rays in air and those given 1,600 r than there was in the corresponding germination percentages. Thus, germination dropped only 6 percent as the irradiation increased from 400 r to 1,600 r, while the growth was reduced by 96 percent.

It might easily be possible that a vacuum could modify X-ray effects by any one of several indirect influences, such as injury to cells from lack of oxygen; removal of water from the seeds as it vaporized in the reduced pressure during evacuation, making them less susceptible to X-ray injury; or the change in gaseous pressure. To test the effect of evacuation itself on germination and chromosome injury, a test-tube containing 20 seeds was attached to a vacuum pump which was left running for three hours. The seeds were then germinated in Petri dishes, and root tips were taken for cytological observations. Both the treated seeds and a control showed 100 percent germination. No chromosomal aberrations were observed among 110 cells examined from the vacuum-treated seeds. Additional proof that evacuation alone did not cause observable deleterious effects is evident in tables 1 and 2 where the seeds subjected to a

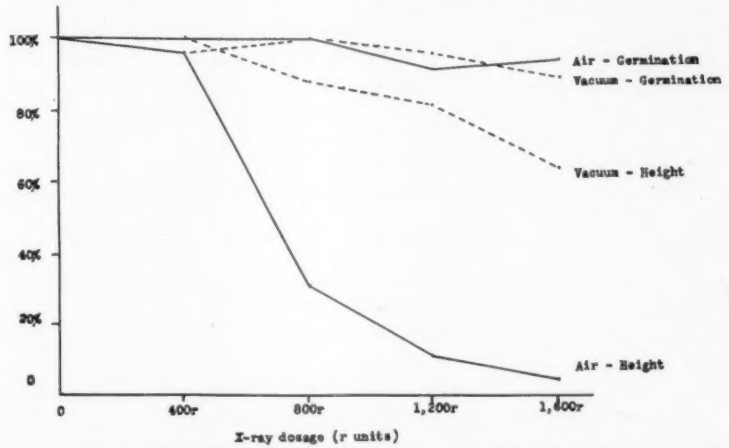

FIGURE 2.—A graph comparing the effects of X-raying germinating barley seeds in air and in a vacuum as measured by germination and growth (height).

vacuum grew about as well as the controls. Keeping seeds in a vacuum for a time might reduce the amount of moisture in them, and as a result make them less susceptible to X-ray injury than seeds that had been soaked an equivalent amount of time and X-rayed in air. There are two reasons for doubting that the vacuum removed enough water to make any significant difference in the

results obtained: 1) in all of these experiments the test-tube containing the seeds was attached to the vacuum pump no more than five minutes while it was in operation, and 2) unpublished data of SMITH showed that keeping dormant seeds of wheat and barley in a vacuum for three hours removed no more than 0.25 percent by weight of moisture. Finally, data from this same paper indicated that pressure alone within the tubes was not the controlling influence on the X-ray effects. This conclusion was suggested by the fact that replacing the air with nitrogen resulted in reduced injury symptoms comparable with those obtained from X-raying seeds in a vacuum. Moreover, a limited amount of data suggested that replacing air with pure oxygen at increased pressures did not increase the injury over that resulting from irradiation in air at normal pressures.

An effort was made to determine the lethal dosages for germinating seeds irradiated in air and in a vacuum, and to determine the dosages given to seeds treated in air that would be equivalent to dosages given seeds treated in a vacuum. The tests are incomplete, but limited observations indicated that the lethal dose for seeds treated in air was approximately 4,000 r, whereas seeds treated in a vacuum survived 5,200 r.

B. The Effect of Irradiation in Air and in a Vacuum on Mutation Rate

In order to compare the mutation rates of seeds irradiated in air and in a vacuum, several samples of 100 or 150 seeds each were irradiated in air simultaneously with numerically equivalent samples irradiated in a vacuum. The treatment was applied in April, 1947, and the seeds were then grown in the field. Whole plants were harvested by pulling in late July. Table 3 presents the results on seedling mutation rates determined by growing head progenies of these plants in the greenhouse in the winter of 1947–48.

The mutation rate obtained from irradiating seeds in air was 3.4 percent as compared with 2.1 percent for those irradiated in a vacuum. The standard

TABLE 3

A comparison of the effects of X-raying (1,200 r) germinating barley seeds in air and in a vacuum as measured by mutation rate and related data.

	TREATMENT		
	CONTROL	X-RAYED IN AIR	X-RAYED IN A VACUUM
Number of seeds treated	500	2,000	2,000
Seeds survived (Percent)	94	60	79
Weight at harvest (Total)	22,400g	36,625g	54,975g
Weight per plant (Average)	47.8g	30.3g	34.6g
Number of head progenies tested		3,425	3,309
Number of progeny per head (Average)		30.7	31.0
Number of mutants (Total)		118	70
Heads with mutants (Percent)		3.4	2.1

Standard error of difference (3.4−2.1) = 1.3±0.4%

error of the difference was 0.4 percent, indicating that the mutation rate in seeds X-rayed in a vacuum was probably significantly lower than the rate in seeds given a simultaneous irradiation while in air. It is apparent that for some reason the vacuum gave a measure of protection to the seeds against mutation, stunting, and reduction in germination.

The types of mutants and the proportions of these types resulting from irradiation in air and in a vacuum are of interest (table 4). Mutations involving self-color changes ranged from white through yellow to yellow-green. Variegated colors included transverse zoning, horizontal striping, and virescence. Though less common in occurrence, other mutant types such as shriveled (necrotic) bands, shriveled tips, or variations of these, appeared. In general, there was a slightly greater percentage of the more extreme types of mutants,

TABLE 4

A comparison of the proportions and types of seedling mutants obtained from X-raying (1,200 r) germinating barley seeds in air and in a vacuum.

FROM SEEDS IRRADIATED IN	MUTANTS (NUMBER)		PERCENT OF TOTAL		FREQUENCY[1] (PERCENT)	
	AIR	VACUUM	AIR	VACUUM	AIR	VACUUM
Mutants (types)						
White	71	30	60	43	15	12
Yellow	11	4	9	6	15	13
Yellow-green	13	15	11	21	21	8
Virescent	10	8	9	11	15	17
Miscellaneous.						
Striped	3	8	3	11	5	7
Banded shrivel	1	4	1	6	25	16
All others	9	1	8	1		
Totals	118	70				

[1] Average percent of mutant seedlings per head progeny.

such as white or yellow seedlings among the progeny from parents X-rayed in air, whereas less extreme mutant types such as yellow-green seedlings seemed to form a greater proportion of the types obtained from seeds exposed while in a vacuum. For example, white seedlings were found to compose 60 percent of the mutants from seeds X-rayed in air and only 43 percent from seeds treated in a vacuum, while, conversely, yellow-green mutants composed 11 percent of the total from irradiation in air and 21 percent from irradiation in a vacuum. Also, there was a slight indication that more seeds per head gave rise to mutant seedlings in those spikes from seeds irradiated in air than from those X-rayed in a vacuum. However, it is apparent from table 4 that there were not enough mutants in most comparisons to give more than suggestive results.

C. The Effect of Irradiation in Air and in a Vacuum on Chromosomal Aberrations

In tables 5 and 6 are summarized the results obtained from cytological examinations of root-tip cells. It is evident that the frequencies of bridges found

TABLE 5

A comparison of the frequencies of chromatinic bridges in cells of root tips grown from germinating barley seeds X-rayed (1,200 r) in air and in a vacuum.

TESTS MADE	AGE OF ROOT TIPS (AFTER RAYING)	TOTAL NUMBER OF CELLS EXAMINED		NUMBER OF BRIDGES PER CELL		STANDARD ERROR OF DIFFERENCE	RATIO OF BRIDGES PER CELL
		AIR	VACUUM	AIR	VACUUM		AIR : VACUUM
Test No. 1	24 hours	99	100	.24	.08	.051	3:1
Test No. 2	30 hours	100	83	.44	.08	.051	5:1
Test No. 3	29 hours	37	27	.19	.07	.077	2:1
Test No. 4	26 hours	16	47	.37	.04	.031	7:1
Totals		252	257				
Weighted average							4.7:1

in cells from root tips of seeds X-rayed in air consistently exceeded the frequencies found in cells from root tips of seeds given the X-ray treatment while in a vacuum. Also, it may be pointed out that there was a greater difference between the frequencies of bridges obtained from treatments in air and in a vacuum than was obtained between the mutation rates. In some cases (table 6), the frequency of bridges from root tips of seeds X-rayed in air was seven times the frequency of bridges found in cells X-rayed in a vacuum, whereas the mutation rate for those seeds treated in air was only 1.6 times the frequency obtained from seeds irradiated while in a vacuum.

TABLE 6

A further comparison of the frequencies of chromatinic bridges in cells of root tips grown from germinating barley seeds X-rayed (1,200 r) in air and in vacuum.
(Summary of four tests distributed over a period of three months.)

TREATMENT	TOTAL NUMBER OF CELLS EXAMINED	FREQUENCIES OF CELLS WITH INDICATED NUMBERS OF BRIDGES					BRIDGES PER CELL (AVERAGE)	STANDARD ERROR OF DIFFERENCE
		0	1	2	3	4		
Control	71	71					0	
Evacuated only	147	147					0	
X-rayed in air	252	202	24	22	3	1	0.30	0.03
X-rayed in a vacuum	257	242	12	1	2		0.08	

DISCUSSION

The relation of atmosphere to the biological effects of X-rays is of interest because it may throw some light on the mechanism by which short-wave radiations produce biological effects. It was hoped that these experiments would help distinguish between the belief of GRAY (1942), LEA (1947 p. 342) and other workers, that injury and death resulting from irradiation are due primarily to genetic aberrations, and the possibility, as suggested by KEMPTON

and MAXWELL (1941), among others, that injury and death may be caused by extra-chromosomal changes. The present results and those of MOTTRAM (1935), SMITH (1946), and THODAY and REED (1947) suggest that X-ray effects (perhaps both chromosomal and extra-chromosomal), may be modified by external conditions, but they do not distinguish between the importance of genetic and physiological factors in biological effects of X-rays.

The results herein reported show clearly that, if the seeds are not in air when X-rayed, they are damaged less (physiologically, genetically, and cytologically) than if they had been in the presence of air when X-rayed. Apparently, a vacuum either exerts a protective influence by keeping the rays from reaching the seeds or there is in the air some component that, in conjunction with irradiation, or activated by it, augments the damage. The first possibility is highly improbable, and the results obtained by MOTTRAM (1935), SMITH (1946 and unpublished), and THODAY and REED (1947) seem to point to the same conclusion: that something in the air, possibly oxygen, increases the injury from irradiation. One hint as to the possible mechanism by which atmosphere may influence X-radiations has been advanced by SKOOG (1935). He showed that auxins are destroyed when X-rayed in the presence of air and presumably this results from an interaction between the X-rays, some component of the air, and the growth hormones—perhaps an oxidation reaction. It is also possible that enzyme inactivation by X-rays (LEA 1947) may be a contributing factor.

There is evidence (STEINITZ 1943) that the absence of oxygen for four days has deleterious effects on barley seedlings. But the data herein show just the opposite trend, so this effect can probably be ruled out in the tests reported here for the short time that the seeds were without oxygen. In spite of the fact that STEBBINS and STEINITZ (1939) had shown that the absence of oxygen in the surrounding atmosphere caused chromosomal aberrations in barley seedlings, THODAY and REED (1947) substituted nitrogen for oxygen or air while roots were being irradiated and obtained reduced frequencies of chromosomal aberrations resulting from the irradiation. Presumably the deleterious effect from keeping seedlings in a nitrogen atmosphere could be neglected since the seeds were not exposed for long periods of time.

Many factors of various natures have been reported to affect the biological effects induced by a given dose of X-radiation. Perhaps the factor that has received most attention has been temperature, about which reports are at variance. KEMPTON and MAXWELL (1941) reported that the maximum sensitivity of air-dried maize seeds to X-rays existed between 0°C and room temperature, with a reduction in sensitivity correlated with either a decrease or increase of temperature outside of this range. (The temperature was maintained during irradiation.) Even when the seeds were held at the temperature of liquid air during treatment the sensitivity was decreased. However, MAXWELL, KEMPTON, and MOSLEY (1942) reported that when cold was applied after irradiation, or heat applied before, the sensitivity of seeds of maize to X-ray injury was increased. Working with the lower side of the temperature scale, TASCHER (1929) and STADLER (1931) reported that extreme cold ob-

tained by packing seeds in solid carbon dioxide reduced sensitivity of dormant barley seeds to X-radiation. Although STADLER (1930) found that temperature had no effect on the production of mutations by X-rays, SMITH and CALDECOTT (1948) observed that the mutation rate of dormant seeds resulting from X-radiation was increased with pre- or post-treatments with heat. These same authors found that the frequency of chromosomal bridges was reduced by application of sub-lethal heat treatments to barley seeds before or after irradiation. These results support evidence of SAX and ENZMANN (1939) who noted a decrease in frequencies of structural changes with an increase in temperature during and after irradiation. The temperature was thought not to have any effect on the actual production of chromosomal breaks, but only on the recombination of broken chromosome ends. FABERGÉ (1940a) stated that high temperature with irradiation reduced the number of fragments. CATCHESIDE *et al.* (1946b) showed that though the number of chromatid breaks (a break in only one of the chromatids of a chromosome) was slightly correlated with an increase in temperature accompanying irradiation, the number of iso-chromatid breaks ("a fracture of a whole chromosome into two parts, both chromatids having been broken at apparently the same locus," CATCHESIDE *et al.* 1946a) was definitely affected by an increased temperature. SAX (1947) found that low temperatures during irradiation increased the frequency of chromosomal aberrations, whereas low temperatures before or after X-ray treatment had no effect.

A second factor that affects biological effects of X-radiation, and about which quite a lot of information has been accumulated, is moisture content. GELIN (1941), FRÖIER and GUSTAFSSON (1941), STADLER (1930), TASCHER (1929), and many others have noted that increasing the moisture content of seeds increases the sensitivity of those seeds to X-ray injury. It has been observed also by GELIN (1941) and others that increased moisture content is correlated with increased frequency of chromosomal aberrations from a given dose of X-rays. Similarly, STADLER (1930, 1931) and GUSTAFSSON (1947) observed that a higher mutation rate was obtained per r unit applied to germinating seeds as compared with dormant seeds.

It has been found that other wave lengths of energy modify the effects of X-rays. HOLLAENDER and SWANSON (1947) reported that a pretreatment of Aspergillus and Trichophyton spores with near-infrared rays increased the frequency of X-ray-induced mutations, whereas infrared alone produced no mutations. KAUFMANN and GAY (1947), summarizing the results from a number of studies by KAUFMANN and several co-workers, reported near-infrared radiation, applied before an X-ray treatment, increased the frequency of chromosomal rearrangements but had no significant effect on the production of recessive, sex-linked lethal mutations. On the other hand, a summary of the work of DEMEREC *et al.* (1942) in which male flies of *Drosophila melanogaster* were exposed to infrared or ultraviolet radiation between two X-ray treatments, showed that the yield of chromosomal structural changes was reduced even though infrared or ultraviolet had no effect when used alone. SWANSON (1944) found that, within limits, an ultraviolet treatment of Tradescantia

pollen tubes before or after X-radiation led to a reduction in the number of chromosomal breaks from a given X-ray dosage.

Additional factors which have been reported to modify X-ray effects may be mentioned briefly. In addition to the observations reported in this paper, MOTTRAM (1935), SMITH (1946), and THODAY and REED (1947) have shown that atmosphere has an influential role on the injury, genetic, and cytological effects of X-radiation. The degree of injury resulting from irradiation has been reported by FABERGÉ (1940a), TASCHER (1929), and SAX (1941b) to be influenced by fractionating the dose, though there have been other reports to the contrary. EBERHARDT (1939), cited by FRÖIER, GELIN, and GUSTAFSSON (1941) presented data indicating that the time factor, or number of r units per minute has some bearing on the degree of X-ray lethality and visible changes. SAX (1941a) and CATCHESIDE *et al.* (1946b) obtained results on the frequency of chromosomal aberrations similar to the findings of EBERHARDT. LEA (1947, p. 145) on the other hand, noted no evidence that the time factor influenced the X-ray-induced mutation rate. BRUMFIELD (1943) suggested that the frequency of chromosomal aberrations and the intensity of radiation may be directly correlated because of the fact that the number of illegitimate fusions depends on the time "breaks remain open." A second factor which facilitates non-homologous pairing or interchanges is the amount of movement of the chromosomes during irradiation, a fact which is supported by the evidence of SAX (1943) that centrifugation during X-irradiation of Tradescantia microspores resulted in a higher frequency of aberrations. FRÖIER, GELIN, and GUSTAFSSON (1941) interpreted EBERHARDT'S results as indicating a difference in effect between hard and soft rays, although STADLER (1930), FABERGÉ (1940b), and a number of other workers have found no such difference within rather wide limits of wave lengths.

Two chemicals which have been reported to modify the effects of X-rays are colchicine and ammonia. BRUMFIELD (1943) observed that onion root tips treated with colchicine before irradiation had one third as many aberrations as those irradiated without colchicine treatment. It was suggested that probably colchicine reduced the movement of the chromosomes in prophase so that, following breakage, restitution rather than rearrangement was favored. MARSHAK (1938a, b) reported that a dilute solution of ammonia applied to onion seedlings before irradiation decreased the number of aberrations appearing three hours later at anaphase.

The cytological composition of the cell influences the reaction to X-rays. Genes themselves may control X-ray susceptibility. SMITH (1942 and unpublished) found evidence of a gene for X-ray sensitivity in *Triticum monococcum*. There is an increase in chromosomal aberrations (bridges, translocations, and irregular cell divisions) associated with an increase in number of genoms (FRÖIER, GELIN, and GUSTAFSSON, 1941; GUSTAFSSON 1947; SMITH 1943 and 1946), though these aberrations prove less likely to be lethal when there are more chromosomes present.

There is some evidence of differences among chromosomes, and among stages in the life cycle of the same chromosome or organism, in their reactions

to X-rays. BAUER, DEMEREC, and KAUFMANN (1938) presented data indicating that a given dose of X-radiation applied to male flies caused fewer breaks in the Y than in the X chromosome of Drosophila, in a ratio approximating 80:100. There is variation in X-ray sensitivity with respect to the stage of mitosis as shown in the frequency of chromosomal aberrations (SAX 1938; SAX and ENZMANN 1939; BRUMFIELD 1943). Increased sensitivity is correlated with the phase of greatest chromosome activity. Consequently, SAX (1938) found meiotic prophase to be ten times more susceptible to X-ray-induced aberrations than the meiotic resting stage, and mitotic prophase to be two times more susceptible than its resting stage. In addition, sensitivity varies with the life cycle (GOODSPEED and UBER 1939) and with the age of the tissue (GUSTAFSSON 1947 and SAX 1942) which to some extent may be associated with the frequency of mitotic activity.

Delaying germination of irradiated onion seeds increased the frequency of aberrations (due possibly to the same physiological effect that age has), but had no effect upon the germination of irradiated onion bulbs (SAX 1941b).

There are still other factors or conditions that influence the biological reactions to X-radiation. For example, dormant onion bulbs are approximately ten times more susceptible to the same dose of X-rays than are dry onion seeds (SAX 1941b). GUSTAFSSON (1947) reported large seeds of barley and wheat capable of tolerating a heavier dose of irradiation than smaller seeds were able to. FRÖIER and GUSTAFSSON (1941) and FRÖIER, GELIN, and GUSTAFSSON (1941) reported that the hulls of "hulled" types of Avena and Hordeum gave seeds some protection against injury from X-radiation. Among seeds of different plants there is wide variance in sensitivity to X-rays (GUSTAFSSON 1947 and TASCHER 1929) and according to FABERGÉ (1940b) "Tradescantia chromosomes behave differently when X-rayed than do those of Drosophila."

Chromatinic bridges at mitotic anaphase presumably result from the fact that a chromatid has two kinetochores instead of one. Irradiation may produce changes that result in chromatids with two kinetochores in at least three ways: 1) two homologous or non-homologous chromosomes or chromatids may break forming four fragments; the broken ends of the two fragments containing kinetochores may join; 2) both chromatids of a chromosome already split into two strands may break with subsequent joining of the broken ends of the two fragments containing kinetochores; or 3) a single strand may break and subsequently split into two chromonemata whose broken ends may join forming a fragment with two kinetochores. In any case, the result is a chromosome (chromatid) with two kinetochores and one, or two akinetic fragments. A bridge is formed if the two kinetochores become destined (presumably by chance if they are not close together) for opposite poles.

In these studies there has been some correlation between injury symptoms, genetic effects, and chromosomal aberrations resulting from X-raying seeds of barley. The correlation between frequencies of genetic effects, as measured by seedling mutation rates, and chromosomal aberrations, as measured by chromatinic bridges was not close. This was pointed out above where it was noted that the mutation rate from X-raying seeds in air was 1.6 times the rate ob-

tained by irradiating seeds while in a vacuum; but the frequency of chromatinic bridges in root-tip cells from seeds X-rayed in air averaged 4.7 times the frequency of those similarly treated in a vacuum. There are two plausible explanations for the contrast between the result obtained on bridge and mutation frequencies: 1) It is probable that cells with aberrant chromosomes either fail to survive or produce smaller proportions of the plant than do cells with normal chromosomes changed only by a "point mutation." Since chromosomal aberrations that lead to bridge formation at mitotic anaphases tend to be eliminated in the ontogeny of plants, and since chromosomal aberrations increase with dosage of X-rays at a different rate than does mutation frequency, a doubling of the mutation rate would not necessarily be expected to accompany a doubling of the frequency of chromatinic bridges in root-tip cells. 2) It might be that the chromosomal aberrations and mutations are not caused by the same mechanism. The evidence on this point is not conclusive.

SUMMARY

1. Barley seeds X-rayed while in a vacuum consistently showed better germination and growth and a lower frequency of chromosomal aberrations than seeds X-rayed in air. In one test the seedling mutation rate from tests of plants grown from germinating seeds X-rayed in a vacuum was 2.1 ± 0.32 percent as compared with 3.4 ± 0.35 percent for seeds X-rayed in air. This difference was slightly more than three times the standard error.

2. Root-tip cells from seeds irradiated in air averaged 4.7 times as many chromatinic bridges as root-tip cells from seeds irradiated in a vacuum.

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GENETIC RECOMBINATION BETWEEN HOST-RANGE AND PLAQUE-TYPE MUTANTS OF BACTERIOPHAGE IN SINGLE BACTERIAL CELLS[1, 2]

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WE HAVE previously shown that any two of several independently arising plaque-type (*r*) mutants of the bacterial virus *T2H* interact with each other, in bacterial cells infected with both, to give rise to wild type and double mutant genetic recombinants (HERSHEY and ROTMAN 1948). In this paper we describe comparable interactions between host-range and *r* mutants of the same virus. The experiments furnish new information because it has proved possible to count the numbers of all four types of virus found in yields from the mixedly infected bacteria.

MATERIALS AND METHODS

The types of viral mutant to which we shall refer in this paper may be summarized in terms of the mutational pattern illustrated in fig. 1. In this diagram, *h* refers to a host-range mutant, *r* to any one of the rapidly lysing mutants (HERSHEY and ROTMAN 1948), and *m* ("minute") to a mutant not previously described which is characterized by a very small haloless plaque. The *h* mutant is one which forms plaques identical in appearance and number on typically sensitive strains of *Escherichia coli*, and on an indicator strain (No. 2 B/2H, 2K) resistant to h^+ forms of the virus (HERSHEY 1946a). All the steps indicated in the diagram by arrows can be observed either as spontaneous mutations, or by making the appropriate crosses. Only one example of the mutant *m*, obtained by crossing wild type with an *rm* arising in a stock of the mutant *r13*, has been studied. The plaques of *m* and *rm* are different, but are not easily distinguishable, as shown in the photograph (fig. 2).

In principle, the experimental technique we have to describe is very similar to that of genetic crossing, and will be referred to in this paper in genetic terms. One starts with a pair of mutants, each corresponding to a mutant haploid germ cell differing from wild type by a different unit change. Bacterial cells are infected with both members of the pair, and during viral growth the pair interact to produce viral progeny corresponding to germ cells of a new

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generation, but now including some individuals differing from wild type by both unit changes, and other individuals differing from wild type not at all.

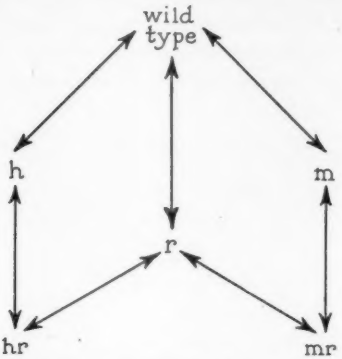

FIGURE 1.—Mutational pattern of the bacterial virus *T2H*.

The analogy to other genetic recombinations is obvious, and it is natural to look for a common mechanism.

The procedure of making a cross consists essentially in infecting a measured number of growing bacteria with larger measured numbers of two kinds of virus, diluting the culture before lysis begins to prevent readsorption of viral progeny to bacteria not yet lysed, and plating samples of the total yield of

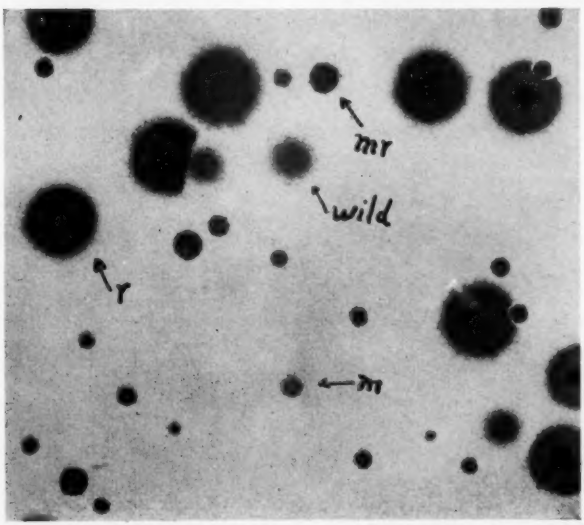

FIGURE 2.—Progeny of the cross *m*×*rI*.

virus for a differential count of its component types. This procedure was first used, for another purpose, by DELBRÜCK and LURIA (1942).

The advantage of the $h \times r$ cross is that all the genetic types of virus to which it gives rise can be recognized in a single plating on a mixture of bacterial strains (fig. 3). This makes possible the analysis of viral yields from single bacterial cells. For this purpose the procedure already described is modified

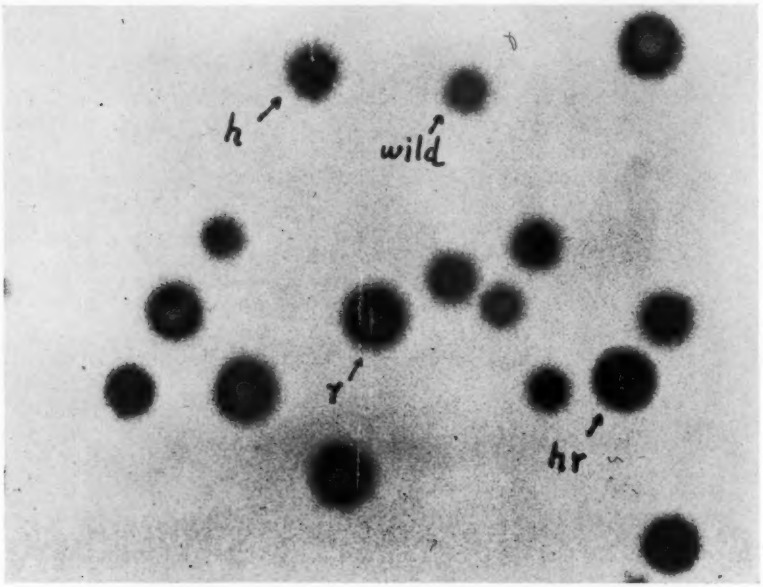

FIGURE 3.—Progeny of the cross $h \times r1$ plated on mixed indicator. The acentric clearings in the h^+r plaques result from secondary h mutations.

by increasing the factor of dilution to obtain only one infected bacterium to about three ml of nutrient broth. The culture is then divided up into samples of one ml, most of which will contain either no bacteria at all, or a single one. A large number of these samples are plated out after the bacteria lyse, and the elementary yields, averaging about 500 particles of virus in our experiments, are analyzed in toto. Both the mixed indicator method for differential counting of viral mixtures, and the single burst technique as here employed, were first used by DELBRÜCK (1945a, b).

The remaining portion of this discussion of materials and methods is of technical interest only.

Viral stocks are prepared by seeding nutrient broth cultures of *E. coli* strain S with material taken directly from a single plaque. Stocks of wild type and h mutant usually have titers exceeding 10^{10}/ml; r mutant titers are always a

little less. Many of the stocks, particularly of the *r* mutants, contain unknown substances inhibiting adsorption of the virus if the dilution into bacterial suspension is less than 1:100 or so. There is no difference in the adsorption of different stocks at high dilutions of the virus. If a mixed infection is attempted with an *r* stock which contains inhibitor, and an *h* or wild type stock which does not, the adsorption of both viruses in the mixture is prevented equally. No undesirable disturbance of the relative multiplicity of infection in mixtures is therefore encountered. To obtain satisfactory levels of adsorption, all *r* mutant stocks are sedimented in a refrigerated International centrifuge with Multispeed head, and resuspended in a solution containing 1 percent Bactopeptone and 0.5 percent NaCl. Very little loss of virus occurs during sedimentation, and probably no permanent aggregation, since sedimented mixtures of *r* and wild type virus do not yield mixed plaques. The resuspended virus is stable for at least several weeks.

To make a cross, a two hour culture of *E. coli* strain H in nutrient broth, containing 2×10^7 bacteria per ml, is infected at 37°C in an aerated culture tube (the "adsorption tube") with $\frac{1}{10}$ volume of a mixture of diluted viral stocks containing 2×10^9 plaque forming particles of each kind per ml. After five minutes, during which equal numbers (about 50 percent) of each virus are adsorbed, a 10^4 dilution is made into broth (the "growth tube") for further incubation, and a second diluted sample is spun for the assay of unadsorbed virus. Sixty minutes after infection, an assay from the growth tube gives the average yield of virus from about 40,000 mixedly infected bacteria.

For the single burst experiments, an additional dilution from the adsorption tube into antiserum to neutralize the unadsorbed virus (DELBRÜCK 1945c), is made at the end of the adsorption period. Five minutes later, a further dilution from the antiserum tube is made into broth to contain about one infected bacterium per three ml. Before the 20th minute after infection, samples measuring one ml are distributed into a series of small tubes. The virus yields in these tubes are assayed by plating 0.3 ml 60 minutes or more after infection. The remainder of each sample, excepting those containing no virus or unmixed yields, is plated on two additional plates the next day. About 10 percent of each sample is mechanically lost. An important feature of these experiments is the guard against contamination of materials provided by the fact that about $\frac{3}{4}$ of the samples contain no virus, whereas the remainder contain more than 100 particles.

Viral yields from the growth tube are plated on sensitive bacteria (strain S), on the indicator strain (No. 2 B/2H, 2K), and on a mixture containing one volume S and two volumes indicator (day-old broth cultures). On the mixed indicator all four types of virus can be recognized (fig. 3), and their sum equals the count on S. Mixed indicators plates always show a few doubtful plaques which can be identified only by sampling and retesting, but their number is too small to be of importance, and mixtures of pure stocks can be counted with satisfactory accuracy. The counts on the single indicator, giving only the *h* mutants, are also satisfactory with mixtures of pure stocks. These counts tend to be low, however, for mixed yields of *h* and h^+ virus. The cause

of this "mixed indicator effect," similar to that observed by DELBRÜCK and BAILEY (1946), remains obscure. It does not appear to be the result of segregation of multiple *h* factors, because no intermediate genetic types can be found in crosses between *h* and wild type. In *h*×*r* crosses, it affects equally counts of parental and recombinant virus.

The validity of the mixed indicator count itself rests on three lines of evidence. First, plaques sampled and retested always conform to the genetic type deduced from inspection. Second, the ratio of *h* to h^+ virus in the yield following mixed infection, measured by the mixed indicator count, is the same as the corresponding ratio of infecting viruses, with minor exceptions to be mentioned below. Third, the yield of the two recombinants in either *h*×*r* or *hr* × wild type crosses is very nearly equal, and the slight bias (actually of doubtful significance) correlates not with the *h*, but with the *r* pair of alleles.

For these reasons, and because of its statistical efficiency, only the mixed indicator plating is used for counting single bursts. The reproducibility of the counts so obtained may be judged from the examples shown in table 1.

TABLE 1

Mixed Indicator Counts of Viral Types in the First Eight Bursts Examined from the Cross h×r7.

The counts shown are for 3 aliquots of 0.3 ml from each tube. The volumes are not measured very accurately, owing to the effort made to plate the entire sample. In computing results, it was assumed that totals of counts for each tube represented 90 percent of the actual virus content, 10 percent of the fluid being lost mechanically.

TUBE	h^+r^+	hr^+	h^+r	hr	TUBE	h^+r^+	hr^+	h^+r	hr
2	9	57	46	19	35	21	80	71	23
	9	77	56	20		24	74	65	18
	11	50	47	18		14	68	39	15
10	18	17	95	13	36	9	136	30	9
	10	14	73	8		7	100	22	7
	10	22	118	15		14	120	18	9
26	9	84	51	8	38	9	139	30	9
	10	124	53	5		9	130	19	13
	8	78	34	7		4	102	31	4
34	2	20	58	17	40	1	24	54	1
	6	26	78	32		3	38	83	10
	6	33	55	16		3	24	66	7

The nutrient broth referred to above is composed of Bacto-peptone 10 g, Bacto-beef extract 3 g, NaCl 5 g, glucose 1 g, per liter distilled water. The pH (unadjusted) is about 6.8. An occasional batch of broth prepared according to this formula proves unfavorable to the adsorption of the virus.

Nutrient agar plates are poured with a minimum of 35 ml per 9 cm Petri dish of Bacto-agar 10 g, Bacto-Tryptose 10 g, NaCl 8 g, sodium citrate crystals 2 g, glucose 1 g, per liter distilled water. The pH of the agar is adjusted to 6.8 to 7.0. Contrary to an early experience, we have recently found that Bacto-Tryptone can be substituted for Tryptose. Poured plates are stored in

the refrigerator. Agar for layer plating has the same nutrient composition, but contains only 0.5 to 0.6 percent agar, the optimal concentration depending on the age of the plates and on other variables.

All platings of virus are made by the agar layer method, by adding an aliquot of virus measuring 0.1 to 0.5 ml and about 0.1 ml of a day old unaerated broth culture of bacteria, to 2 ml of melted soft agar at 45°C, and pouring the mixture over the surface of an agar plate at room temperature. The plates are incubated 18 to 24 hours at 37°C without inverting, during which time fluid collects on the agar surface without undesirable effects if conditions are optimal.

Bacterial counts are made by spreading 0.1 ml aliquots on the surface of dried agar plates.

THE LINKAGE SYSTEM

The mutants *m* and *h* (*minute* plaque and *host-range* modification, respectively) have been crossed with wild type, with the mutants called *r1*, *r7*, and *r13*, and with each other. The crosses with wild type yield only the parental types of virus, confirming that the mutants are unit modifications. The crosses with *r* mutants, and the intercross, extend the linkage system previously described (HERSHEY and ROTMAN 1948) as shown in fig. 4. The *h* locus is closely linked to the locus *r13*; the *m* locus belongs in a third linkage group C.

It should be understood that the diagram of fig. 4 is only a convenient representation of linkage relations whose structural basis remains to be elucidated (HERSHEY and ROTMAN 1948). The question of linear structure will be returned to in the discussion of this paper. At this point we insert an experiment which supports the general interpretation in terms of linkage.

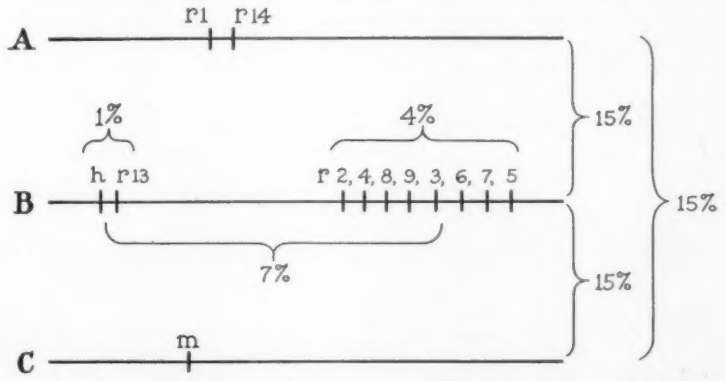

FIGURE 4.—Linkage relations among mutants of *T2H*. The percentages indicate yields of wild type in two factor crosses.

The double mutants *h r7* and *h r13* were isolated by making the respective crosses *h*×*r7* and *h*×*r13*. The three crosses *r7*×*r13*, *h r7*×*r13*, and *h r13*×*r7* were then compared with respect to the yield of r^+ virus, classified without

respect to the host-range character. In each case the yield was the same within experimental error, seven percent of the total virus. This experiment shows that the small yield of recombinants (one percent of wild type) in the cross *h*×*r13* cannot be attributed to suppression of a hypothetical conjugation, which would affect the interaction of *h r7* with *r13* as well, but indicates some kind of linkage between the genetic factors concerned.

AVERAGE YIELDS OF VIRUS IN CROSSES BETWEEN HOST-RANGE AND *r* MUTANTS

Six crosses have been studied by the single burst technique; namely, *h*×*r1*, *h*×*r7*, *h*×*r13* and the corresponding reverse crosses, *h r*× wild type. Each cross was made three to five times for the collection of the single burst data, and each time the viral yield was examined also from a culture tube containing about 40,000 mixedly infected bacteria. The average yields from the culture tubes are summarized in table 2. They show that the *h* locus is very closely linked to *r13* (less than one percent of wild type), and that the linkage relations to *r1* and *r7* are approximately what would have been predicted from this fact, respectively 12 and 6 percent of wild type. According to arguments previously given (HERSHEY and ROTMAN 1948), the factors *h*, *r7*, and *r13* are

TABLE 2

Average Percent Distribution of Viral Types in Yields from about 40,000 Mixedly Infected Bacteria

The results shown are from the same experiments for which single burst data are also reported, except that one growth tube was lost among the crosses *hr7* by wild type. The total multiplicity of infection is about five of each type per bacterium; the adsorption period is five minutes; the total incubation period one hour. The distribution of viral types is computed from the results of mixed indicator platings. The column headed eop(h) gives the efficiency of plating of *h* virus on single indicator as compared with mixed indicator, and illustrates the mixed indicator effect mentioned in Methods. The column headed p(h) gives the percent of virus containing the *h* allele, and shows the effect of selection during growth.

CROSS	NO. OF EXPTS.		h^+r^+	hr^+	h^+r	hr	BURST SIZE	eop(h)	p(h)
h×*r1*	5	input	0	53	47	0		1.0	53
		yield	12	42	34	12	630	0.8	54
hr1×++	3	input	57	0	0	43		1.0	43
		yield	44	14	13	29	680	0.7	43
h×*r7*	4	input	0	49	51	0		1.1	49
		yield	5.9	56	32	6.4	650	0.8	62
hr7×++	2	input	49	0	0	51		1.0	51
		yield	42	7.8	7.1	43	690	0.8	51
h×*r13*	3	input	0	49	51	0		1.1	49
		yield	0.74	59	39	0.94	510	0.8	60
hr13×++	4	input	52	0	0	48		0.9	48
		yield	50	0.83	0.76	48	590	0.7	49

linked to each other, but *r1* is probably situated on an independently exchanging structure.

The results of table 2 show further that the two recombinants appear in equal numbers in any one cross, and that pairs of reverse crosses yield equal numbers of recombinants. It is these relations, which increase the resemblance to simple types of Mendelian segregation, that we wish to examine by the single burst technique. In the remainder of the experimental part of this paper we describe the results of this examination, but limit our comments chiefly to the technical problems encountered. The general implications of the data will be considered in the discussion.

VARIATIONS IN YIELDS OF PARENTAL TYPES OF VIRUS AMONG INDIVIDUAL BACTERIA

Yields of virus from single bacteria show large fluctuations in size (DELBRÜCK 1945b) and, in our experiments, variations in relative yields of the two infecting viruses. The variations in total yield are shown in fig. 5 which includes the complete data for six experiments in which the proportion of multiple bursts is small (100 bursts out of 484 tubes, or 11 probable multiples). The burst sizes range from 150 to several thousand, with a mean of 520, or 470 corrected for probable multiples. The distribution is the same for the mixed bursts, and for the bursts containing only one viral type. Owing to these variations, it is convenient to describe the individual bursts in terms of the fractional yield of the several viral types.

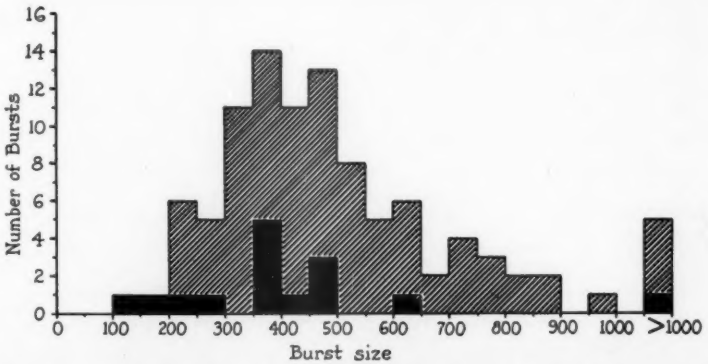

FIGURE 5.—Distribution of total viral yields among 100 single bursts from mixedly infected bacteria. The solidly shaded areas refer to unmixed yields.

Each cross yields four types of virus, wild type, the single mutants *h* and *r*, and the double mutant *h r*, of which two are parental and two are recombinant types. In order to examine the variations in relative yields of parental types independently of variations in yields of recombinants, it is convenient to express the former in terms of the proportion of virus containing a specified allele, viz:

$$p(h) = \frac{n(hr^+)+n(hr)}{n\ (total)} \quad (1)$$

$$p(r) = \frac{n(h^+r)+n(hr)}{n\ (total)} \quad (2)$$

where n indicates the number of the specified viral types, p(h) is the proportion of the yield containing the h allele, and $1-p(h)$, p(r), and $1-p(r)$ are respectively the proportions containing the h^+, r, and r^+ alleles. The fraction p(h) is equal to $1-p(r)$ if the numbers of the two recombinant types are the same, or approximately equal if these numbers are small. A fair idea of the complete distribution of alleles is therefore given by the distribution of p(h) alone. Two examples of this distribution, showing the proportions of the h allele in different single bursts for the crosses $h \times r1$ and $h \times r7$, are given in fig. 6.

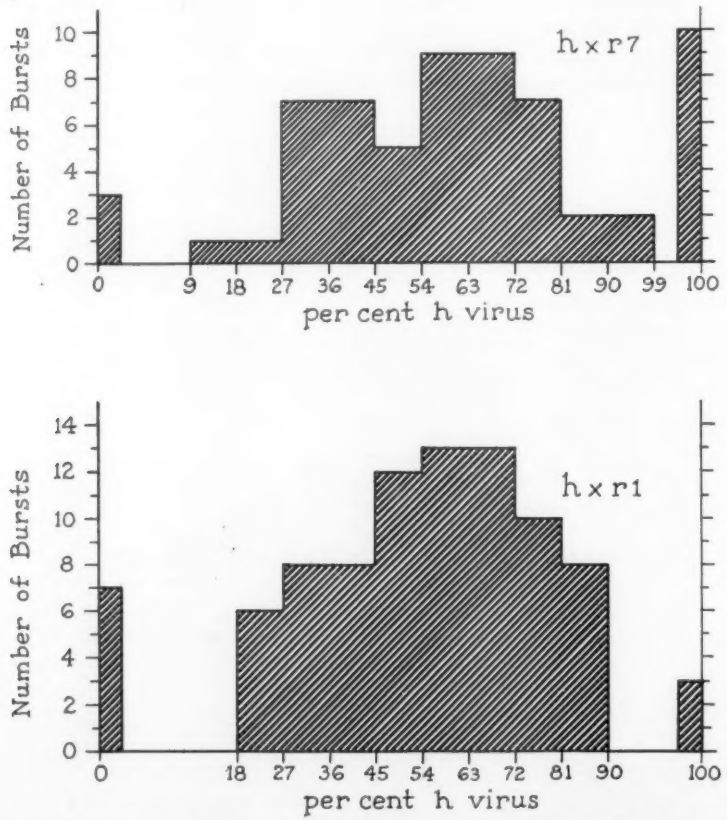

FIGURE 6.—Distribution of proportions of h virus in yields from single bacteria.

The variations shown in fig. 6 are evidently due in part to variations in relative numbers of the two kinds of virus adsorbed, and in part to variations in viral growth. For purposes of comparison, the variation in relative multiplicity has been computed on the assumption of a random distribution of two types of virus over a population of bacteria receiving on the average five particles of each kind. The distribution for this case is approximately that shown in table 3.

TABLE 3

Ideal Distribution of Multiplicities in Mixed Infection

The proportions of bacteria falling into the specified groups classified with respect to relative multiplicity of infection with two kinds of virus have been calculated for random adsorption with average multiplicity of five each of the two kinds.

NUMBERS OF FIRST KIND AS PERCENT OF TOTAL VIRUS ADSORBED	PERCENT OF BACTERIA
0 or 100	0.7 (exact)
1–9 or 91–99	0.2 (approximate)
10–18 or 82–90	2.0 "
19–27 or 73–81	5.1 "
28–36 or 64–72	10.8 "
37–45, 46–54, or 55–63	18.2 "
total	92.2

The distributions of yields actually found (fig. 6) differ from the theoretical distribution of multiplicities in showing a considerably broader spread, and a significant excess of yields containing only one kind of virus. These effects could be due in part to an inhomogeneity of the bacteria with respect to adsorbing power for virus; otherwise they suggest that a bacterium infected with two viruses is somewhat less likely to liberate a given one, than a bacterium infected with that one alone. DULBECCO (1949) has shown that the latter is, in fact, the case.

Another possible contribution to the variations described is connected with the relatively long period (five minutes) allowed for adsorption of virus, which permits some bacteria to be infected with one or more particles of one type of virus considerably in advance of infection by the second. Owing to the slow adsorption of the virus *T2H* and its mutants, the adsorption time cannot be much reduced without reducing the total multiplicity of infection, or introducing excessive amounts of virus. This contribution to the variation in composition of viral yields has not, therefore, been assessed.

COMPETITION BETWEEN VIRAL MUTANTS

The competition between viral mutants expresses itself in two ways; first, by the complete suppression of one virus or the other in mixedly infected bacteria, and second, by excessive growth of one of the two types in bacteria liberating both. These effects are slight among mutants of *T2H* so far examined,

and probably do not influence appreciably the yields of recombinants in genetic crosses, as the following discussion will show.

The two distributions shown in fig. 6 illustrate the competitive relations encountered. In the cross *h*×*r7*, most of the unmixed yields contain the *h* rather than the *r* parental type. Corresponding to this, there is a tendency for the mixed yields to contain an excess of *h* virus. The combined effect is to cause a definite increase in yield of *h* virus at the expense of *r*, as compared with the input proportions. The cross *h*×*r13* also shows these characteristics, the effects being evident in table 2.

In the cross *h*×*r1*, on the other hand, the unmixed yields of each kind are approximately equal in number, and the mean proportion of *h* virus in the mixed bursts, in the total yield, and in the input mixture of viruses is the same. The crosses between wild type and *h r1*, *h r7*, and *h r13* are like *h*×*r1* in this respect, as shown in table 2.

It might be supposed that the suppression of one virus by a second is favored by an excess of the second. This is true only in a special sense, as DULBECCO (1949) has shown, and we have confirmed. An excess of one virus tends to suppress a minority type completely in some bacteria, but there is a compensating excess of this type among the mixed bursts, so that the average proportion of the minority virus in the yield averaged over many bacteria is the same as in the input mixture. This identity has been established with considerable precision for proportions of *r1* between 7 and 50 percent in mixed infection with wild type. The nature of this relationship, which is at first sight perplexing in the case of unequal multiplicity, has been explained by DULBECCO (1949) in terms of a limitation to the number of viral particles which can participate in growth in a single bacterium. If all those viral particles in excess of a certain number attached to the same bacterium fail to grow, and if the excluded ones are chosen at random, the result will be precisely the one described, provided there is no selection during the growth of the successful particles.

It is apparent that with certain viral pairs, the excluding mechanism does not operate at random, or there is continuing selection during growth. Thus *h* mutant slightly suppresses *r7* or *r13*, but not *r1* or wild type. Wild type suppresses *r13*, but not *r1* or *r7* (HERSHEY and ROTMAN 1948). There is no selection with respect to either *h* or *r* factors when wild type is crossed with *h r1*, *h r7*, or *h r13* (table 2).

The competitive relations discussed above are of immediate interest only in the negative sense that they probably do not influence the yields of recombinant virus in crosses. The evidence for the latter conclusion, drawn from data presented elsewhere in this paper, may be summarized as follows: (1) the linkage relations deduced from average yields of virus are the same as those deduced from single bursts selected for equality of yields of the two infecting viruses; (2) in the reverse crosses *h*×*r7* and *h r7* × wild type, one gets within experimental error equal numbers of all four recombinants in spite of the fact that in one case the infecting pair, and in the other the recombinant pair, have unequal excluding power; (3) in all crosses, the distribution of yields of recombinants among single bursts does not show one peak at zero and another

above the mean, as is the case with the yields of a minority infecting type, but shows a single mode slightly less than the mean.

The last two lines of evidence cited seem to show that the principle of limited participation (DULBECCO 1949) referred to above, operates only during the initial stages of infection, or at any rate does not influence the yields of genetic recombinants arising within the mixedly infected bacteria. They suggest further that the *h* mutant is superior to *r7* or *r13* in excluding power only, not as a competititor during actual multiplication.

YIELDS OF GENETIC RECOMBINANTS FROM SINGLE MIXEDLY INFECTED BACTERIA

In order to study the variations in yields of recombinants intrinsic to the recombination process, one would like to exclude as many as possible of the accessory sources of variation. The most important of these are variations in burst size, and variations in the relative numbers of the two infecting viruses adsorbed to individual bacteria. It will be seen presently that effects of variations in burst size can be avoided by the simple expedient of computing proportionate yields of recombinants, these being independent of burst size. The effect of variations in relative multiplicity could be minimized either by going to very small or very large total multiplicities. Low multiplicities are uneconomical, because at multiplicities sufficiently small so that most of the mixedly infected bacteria receive only one viral particle of each type, very few of the test cultures will yield a mixed burst. High multiplicities also introduce difficulties (DULBECCO 1949). We have chosen to use total multiplicities between 10 and 20, within which range the yield of recombinants is constant.

As previously described, the elementary viral yields vary considerably in the relative numbers of the two parental types of virus and, as expected, these variations influence in turn the yields of recombinants. A correction for this source of variation was devised as follows. Assuming that the genetic interaction occurs between unlike viral pairs, and that the composition of the viral yield provides a direct measure of the composition of the intracellular viral population during growth, one computes an interaction coefficient

$$k = p(h)[1 - p(h)] \qquad (3)$$

in which p(h) is given by (1), and k expresses the influence of the composition of the population on the number of unlike viral pairs present in the cell, neglecting effects of genetic recombination.

The coefficient k has a maximum of 0.25 when half the viral yield contains the *h* allele. Dividing the proportions of recombinants by 4k serves therefore as a correction for inequality of yields of the parental viruses. This correction is ambiguous only for bursts in which the yields of the two recombinants are large and unequal, and bursts from which either recombinant is absent.

A summary of the single burst data is given in table 4, which includes the mixed bursts only. The bursts have been separated into the classes $k \geqq 0.21$ and $k \leqq 0.20$, to show the effect of the correction described above. It will be seen that the uncorrected mean proportion of recombinants is larger for the

bursts with the larger k, and that the effect of dividing each proportion by 4k is to make the results homogenous. These facts justify the use of the correction. Its theoretical significance is clarified in the discussion.

TABLE 4

Single burst data for h×r crosses

k = a measure of disproportion between yields of parental types (Eq. 3).
$\bar{x}$ = average yield of the r^+ recombinant as percent of total virus.
$\bar{y}$ = average yield of the r recombinant as percent of total virus.
n = average burst size.
r(x, y) = coefficient of correlation between proportionate yields of the two recombinants.
r(n, x+y) = coefficient of correlation between burst size and sum of proportions of the two recombinants.
The variations of x, y and n/100 shown are standard deviations within the sample. The standard errors of the means are obtained by dividing these by the square root of the number of bursts.
The correction referred to is described in the text.

CROSS	NO. OF BURSTS	k	4k		$\bar{x}$	$\bar{y}$	$\bar{n}$/100	r(x, y)	r(n, x+y)
h×r1	25	≦0.20	0.65	uncorr.	8.9±3.8	12.1±6.2	5.5±2.4		−0.25
				corr.	13.9±5.3	19.0±9.2		0.09	
	52	≧0.21	0.94	uncorr.	15.5±6.9	17.1±7.1	5.8±3.2		0.15
				corr.	16.6±6.3	18.1±7.2		−0.01	
h r1×++	19	≦0.20	0.67	uncorr.	13.6±5.9	13.2±4.4	5.1±1.9		−0.26
				corr.	20.1±7.4	20.8±8.5		−0.27	
	36	≧0.21	0.94	uncorr.	16.5±5.8	17.1±5.6	5.4±3.0		−0.11
				corr.	17.5±6.2	18.2±6.0		0.16	
h×r7	13	≦0.20	0.64	uncorr.	5.7±3.5	6.6±4.6	6.2±1.9		−0.43
				corr.	8.8±4.8	9.9±5.9		0.65	
	35	≧0.21	0.93	uncorr.	6.8±4.2	9.2±4.5	5.9±3.2		0.16
				corr.	7.2±4.3	9.8±4.8		0.53	
h r7×++	17	≦0.20	0.63	uncorr.	6.4±3.1	4.8±2.9	6.4±7.8		−0.45
				corr.	10.0±4.3	8.0±5.0		0.12	
	26	≧0.21	0.95	uncorr.	6.7±3.2	8.9±5.4	5.8±3.7		0.02
				corr.	7.1±3.6	9.3±5.6		0.47	
h×r13	29	≦0.20	0.61	uncorr.	0.5±0.5	0.8±0.8	5.2±2.1	0.07	0.35
	29	≧0.21	0.94	uncorr.	0.9±0.9	1.1±0.9	5.1±2.3	−0.05	−0.30
h r13×++	21	≦0.20	0.69	uncorr.	0.6±0.7	0.6±0.9	4.6±1.4	0.15	0.22
	46	≧0.21	0.94	uncorr.	0.8±0.7	0.7±0.6	4.9±2.3	0.21	0.04

The data of table 4 for mixed single bursts confirm fairly well the average data of table 2, except that the yields of recombinants are somewhat greater owing to the exclusion of the unmixed bursts, and that the yields corrected for unequal growth of parental viruses are higher still.

The chief point of interest is the question of the correlation between yields of the two recombinants in single bursts. This has been measured in terms of the correlation coefficient r (RIDER 1939). This measure varies between −1 and +1, a value near 0 indicating independence of variates, and values near unity indicating negative or positive correlation, respectively. The data of table 4 show clearly that there is no significant correlation between the proportions of the two recombinants in single bursts except for the crosses *h×r7* and *h r7×* wild type. Even for these crosses the correlation is weak and not entirely convincing, especially since the data are not completely unselected (see below).

The correlation between the uncorrected proportions of the two recombinants is shown in the form of scatter diagrams in fig. 7. These data might be expected to show some degree of spurious correlation owing to the fact that bursts with disproportionate yields of the two parental types tend to contain diminished numbers of both recombinants. This tendency can be seen in the

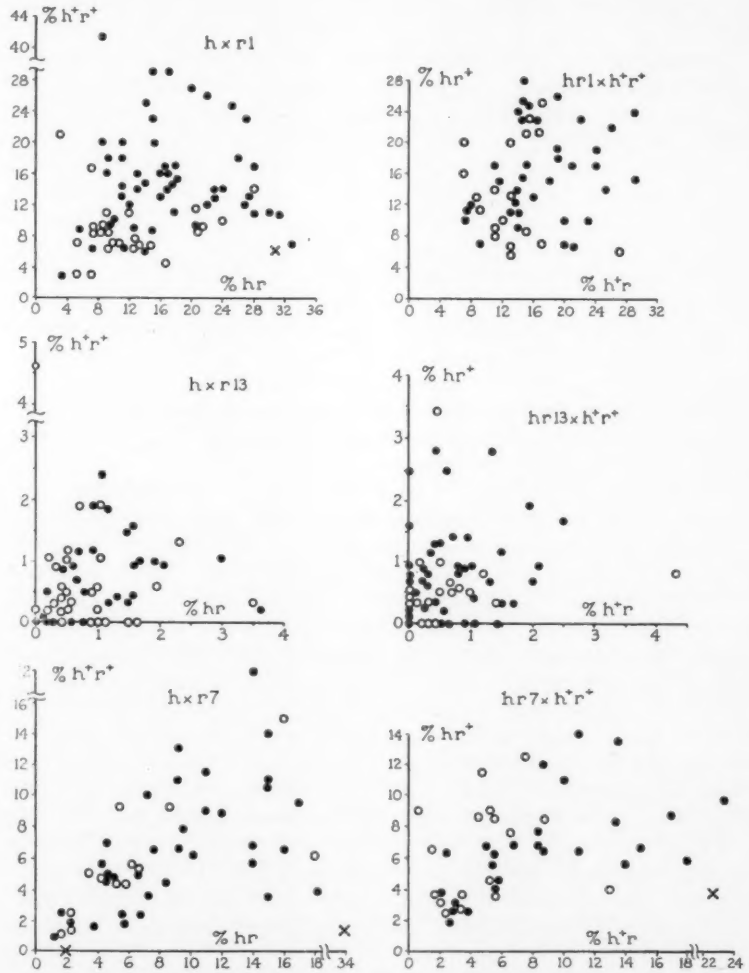

FIGURE 7.—Correlation between proportionate yields of the two recombinants in single bursts. The open circles indicate bursts with disproportionate yields of the two parental viruses. Crosses indicate yields omitted from the data of table 4.

diagrams in which the disproportionate yields ($k \leqq 0.20$) are indicated by open circles. Actually this effect is of minor importance, since the variations due to unknown causes are so much greater than those due to variations in k. Consequently, the uncorrected data lead to the same conclusions as the corrected data; namely, that the proportionate yields of the recombinants are uncorrelated in the crosses *h*×*r1*, *h*×*r13*, and the corresponding reverse crosses, but that there is a weak positive correlation for the crosses *h*×*r7* and *h r7*× wild type.

As mentioned earlier, the correlation data in table 4 are not unselected. The diagrams of fig. 7, however, show all the mixed bursts for the respective experiments, those omitted from the table being indicated by crosses in the diagram. It is plausible that some of the discrepant bursts came from bacteria infected with a spontaneous mutant present in one of the parental stocks of virus. In fact, two bursts from the cross *h*×*r7* were found to contain a large proportion of mutants, *m* in one case and weak inhibitor (HERSHEY 1946b) in the other, which almost certainly arose in this way. The *h* stock used in these crosses contained about 0.1 percent of *h r* virus, so that in crosses with *r* at least one bacterium in 200 was infected with three types of virus, and would be likely to yield an excess of one of the recombinant phenotypes. Unfortunately the recombinant progeny in the exceptional bursts were not checked by crossing with the parental stocks, which should be done in any further experiments of this type. For the present these bursts throw some doubt on the significance of the positive correlation between proportions of recombinants in the crosses *h*×*r7* and *h r7*× wild type.

The results of the cross *h*×*r13* are of special interest because of the small yield of recombinants. The distribution of the bursts with respect to absence of recombinants is shown in table 5. Nine of the 125 mixed bursts fail to show either recombinant, and 31 more lack one recombinant or the other. One can test the hypothesis that the two sister recombinants arise independently as follows. About 20 percent of the bursts fail to show a specified one of the two possible recombinants. If the absence of the one were independent of the absence of the other, about $(0.2)^2$ or four percent of the bursts should show neither recombinant. The number found, 9/125, is larger than this, but not significantly so. Moreover, bursts lacking one recombinant do not show less than the average proportion of the other (table 5). The data evidently fail to exclude the hypothesis of independent origin of the two recombinants, but do not, of course, rule out the hypothesis of reciprocal exchange.

Another question that arises in connection with the data is concerned with the number of genetic exchanges per bacterium. For explicitness, we consider separately the hypotheses of reciprocal and non-reciprocal exchange. If exchanges are reciprocal, the bursts lacking a single recombinant are the result of failure to recognize the few plaques of either type, of losses in the ten percent of each culture not examined, and of unspecified biological accidents. As previously computed, four percent or 5 of the 125 bursts fail to show either recombinant for one or another of these reasons, leaving only 4 without recombinants possibly owing to failure of exchange. This number is too small

if some of the single absences result from erroneous recognition of one recombinant, but is otherwise subject only to its sampling errors. Taken as a measure of failure of reciprocal exchange, the fraction 4/125 implies an average of 3.4 exchanges per bacterium. If, on the other hand, exchanges are not reciprocal, the fraction (20 percent) of the bursts lacking any given recombinant corresponds to 1.7 genetic transfers per bacterium.

These estimates may be too low if conditions vary from bacterium to bacterium in such a way that genetic exchange is suppressed in some bacteria.

TABLE 5

Distribution of Bursts with Respect to Absence of Recombinants

See legend table 4. The proportions of recombinants have not been corrected for disproportionate yields of parental types of virus.

CROSS	CLASS	NO. OF BURSTS	$\bar{x}$	$\bar{y}$	$\bar{n}$	$\bar{k}$
h×*r13*	both absent	2	0.0	0.0	390	0.20
	h^+r^+ absent	10	0.0	0.81	460	0.19
	h r absent	2	2.4	0.0	390	0.19
	total	58	0.72	0.97	520	0.20
h r13×++	both absent	7	0.0	0.0	340	0.21
	$h\,r^+$ absent	8	0.0	0.66	360	0.21
	h^+r absent	11	0.75	0.0	380	0.22
	total	67	0.73	0.64	480	0.22

Yields with small k and small burst size must have this effect, but are evidently not very important in the data of table 5, since the different classes are very similar with respect to k and burst size. In short, it is necessary to conclude that there are at least two or three genetic exchanges per bacterium, independently of the mechanism by which recombinants arise.

A different kind of estimate of the number of exchanges per bacterium is obtained from the number of recombinants actually found. The data are summarized in table 6 in the form of distributions of numbers of the several recombinant types. One finds on the average 3.4 recombinants of any one kind per bacterium. This evidently furnishes an upper limit to the number of exchanges per bacterium, insofar as exchanges yield viable and countable progeny. This result, taken in conjunction with the preceding estimates, leads to several remarkable conclusions.

First, since the two methods of estimation, one minimal and one maximal, yield about the same result, there must be in fact only two or three exchanges per bacterium in the crosses between closely linked factors.

Second, the recombinants must undergo little multiplication after they arise in the cell.

Third, the conditions of viral growth in different bacterial cells must be equally favorable to genetic recombination; otherwise a larger proportion of bursts would fail to show recombinants.

These conclusions are substantially confirmed by the distributions of numbers of recombinants shown in table 6, which are essentially of the Poisson type, with variance only moderately greater than the mean. In other words, the individual particles of any one recombinant must often arise independently of each other in the same bacterial cell, and with equal probability in different bacterial cells. The deviations from the Poisson distribution are nevertheless significant, and can be attributed to a moderate amount of growth of recombinants.

TABLE 6

Distributions of Numbers of Recombinants in Single Bursts from the Crosses h×r13 and h r13×wild type

The Poisson distributions show the numbers of tubes in the various classes expected if there were no growth of recombinants. The distribution with mean 3.4 is appropriate to the hypothesis of reciprocal exchange, and the distribution with mean 1.7 to the hypothesis of non-reciprocal genetic interaction.

NO. OF RECOMBINANTS PER TUBE	NO. OF TUBES FOUND				POISSON DISTRIBUTIONS	
	h^+r^+	$h\ r$	$h\ r^+$	$h^+\ r$		
0	12	4	15	18	2.1	11.5
1	11	11	11	14	7.2	19.6
2	9	8	8	6	12.2	16.7
3	6	9	11	9	13.8	9.5
4	3	7	6	4	11.7	4.0
5	7	3	4	2	8.0	1.4
6	1	2	3	6	4.5	0.4
7	3	6	0	2	2.2	0.1
8	2	2	3	3	0.9	0.0
9	1	2	2	1	0.3	0.0
10–18	3	2	3	2	0.1	0.0
19–27	0	1	1	0	0.0	0.0
28–36	0	1	0	0	0.0	0.0
No. of tubes	58	58	67	67	63	63
Mean per tube	3.1	4.5	3.3	2.8	3.4	1.7
Variance	9.2	27	16	10	3.4	1.7

The conclusion that genetic recombination is not suppressed in some of the bacteria is also supported by the results of the other crosses, in which no bursts yielding both parental viruses and lacking both recombinants were found. Only one burst, from the cross $h\times r7$, failed to show one of the recombinants; it contained 95 percent of the *h* parent.

INDEPENDENCE BETWEEN PROPORTION OF RECOMBINANTS AND BURST SIZE

The data of table 4 do not show any significant correlation between burst size and proportion of recombinants, which means that the number of recombinants must be very nearly proportional to the total yield of virus in single bursts.

This conclusion must be qualified in view of the following considerations.

Some of the tubes contain one or more mixed bursts plus one or more unmixed bursts. Such tubes would tend to show less than average values of k and less than average yields of recombinants together with greater than average burst size. The number of such tubes is about five percent of the total in our experiments and these tend to be concentrated in the class $k \leqq 0.2$. This probably explains the negative correlation between proportions of recombinants and burst size among tubes selected for small k.

A larger proportion of the tubes, between 10 and 20 percent in our experiments, contain two or more mixed bursts. These tubes would tend to have greater than average k and greater than average burst size, but will not show exceptional proportions of recombinants. These tubes have the effect of weakening any correlation that may exist between burst size and proportion of recombinants, especially among the class $k \geqq 0.21$. The data previously considered do not, therefore, exclude the possibility of a weak positive correlation between proportion of recombinants and size of bursts.

Advantage was taken of the finding that high pH reduces size of bursts, to examine the relation between burst size and yield of recombinants in another

TABLE 7

Small Bursts from the Cross h×r7 in Broth of pH 9.0

See legend table 4. The proportions of recombinants have not been corrected for disproportionate yields of parental types of virus. The individual cultures contain an average of about 1.4 mixed bursts.

NO. OF BURSTS	k	$4\bar{k}$	$\bar{x}$	$\bar{y}$	$\bar{n}$	r(x, y)	r(n, x+y)
20	$\leqq 0.20$	0.61	6.3±3.8	10.7±6.2	99±50	0.53	−0.34
34	$\geqq 0.21$	0.93	7.4±3.5	10.5±4.6	123±73	0.61	0.35
54	all	0.81	7.0±3.7	10.6±5.2	114±66	0.56	0.12

way. Since increased pH was found also to cause some of the bacteria to fail to liberate virus, the single burst technique was chosen.

Bacteria were infected in the usual way with mutants *h* and *r7* in broth of pH 6.8. At the end of the five minute adsorption period, and without treatment with antiserum, single burst cultures were prepared after diluting in broth of pH 9.0. Preliminary experiments showed that the yield of virus was complete under these conditions within one hour, and than no inactivation occurred during two additional hours at 37°C, or overnight in the refrigerator. Entire samples were plated on single mixed indicator plates. One successful experiment yielded 66 bursts, of which 54 were mixed, among 104 tubes receiving on the average 1.4 bacteria per tube. Evidently most of the bacteria liberated some virus. The average burst size, after subtracting the virus carried over from the input (totalling 36 particles per tube), was only 114 per tube, or about 70 per bacterium corrected for the probable multiples. The average proportion of recombinants was nevertheless of normal size (table 7).

It will be noticed also that the correlation between the numbers of the two recombinants in these bursts is exceptionally good. The correlation is, however,

slightly exaggerated owing to the fact that the 54 tubes contain on the average mixed yields of virus from about 1.4 bacteria per tube.

THE EFFECT OF A SHORT PERIOD OF ADSORPTION AND LOW MULTIPLICITY OF INFECTION ON THE DISTRIBUTION OF RECOMBINANTS

The following experiment shows that when the multiplicity of infection in the cross *h*×*r7* is reduced from five to about one of each viral type per bacterium, the distribution of recombinants among single mixed bursts is little if any altered.

Six crosses were made in the usual way, except that the period allowed for adsorption was reduced to one minute, without reducing the total input of virus. The amount of virus adsorbed was too small to be measured, but the multiplicity of infection can be estimated from the data given below.

The single burst cultures collected from the six crosses are sufficiently similar to be considered together. The mean number of bacteria per tube for the six sets, determined by colony counts from the growth tubes immediately before adding virus, is 0.23. The mean number of infected bacteria per tube determined by plaque counts of samples taken before lysis is 0.20. The mean number of bursts per tube calculated from the proportion, 141 out of 720, of tubes containing virus is 0.22. The 141 tubes therefore contained about 157 infected bacteria.

From the distribution of viral types among the tubes, namely, 69 containing *h* only, 22 containing *r* only, 50 containing both, and 579 containing neither, one finds the probable distribution with respect to bacteria to be 80 infected with *h* only, 28 with *r* only, 49 both, and 46 neither. The multiplicity of infection is therefore about 1.0 with respect to *h*, and 0.48 with respect to *r*. One can estimate further that about seven of the tubes contained one or more mixed bursts plus one or more unmixed; and that about three contained both *h* and *r* bursts without any mixed bursts. Also, among the mixedly infected bacteria, 45 percent were infected with one particle only of each viral type.

In making the above computations we have neglected the probability that the *h* mutant suppresses the growth of *r* in some bacteria adsorbing both types of virus. The apparent inequality of infection is probably due in some part to this effect. However, in other experiments with low multiplicity of infection with *h* and *r7* designed to check this point, the split into *h*, *r*, and mixed yielders was nearly equal. It seems likely, therefore, that in the experiments reported here the two viral types were unequally adsorbed for unknown reasons.

The tubes containing only one viral type may be dismissed by saying that their average content of virus did not differ significantly from that of the mixed yields, and that the *h* and *r* yields per bacterium were the same. There was one exceptional burst containing only *h r*$^+$ and *h r* phenotypes. The characteristics of the remaining 49 cultures containing *h* and *r* virus are summarized in table 8. The data show no unusual features excepting the small burst size, which is a direct effect of the low multiplicity of infection, and the somewhat

small proportion of recombinants, probably due to the appreciable number of superimposed unmixed bursts.

TABLE 8

Single Bursts from the Cross h×r7 with Low Multiplicity of Infection

See legend table 4. The proportion of recombinants have not been corrected for disproportionate yields of parental types of virus. Adsorption time one minute. Multiplicity 1.0 *h* and 0.5 *r* per bacterium.

NO. OF BURSTS	k	$4\bar{k}$	$\bar{x}$	$\bar{y}$	$\bar{n}/100$	r(x, y)	r(n, x+y)
17	$\leqq 0.20$	0.59	5.3±4.0	4.5±3.7	2.9±1.6	0.38	−0.17
32	$\geqq 0.21$	0.95	4.4±3.1	6.1±5.4	3.5±2.0	0.20	−0.33
49	all	0.84	4.7±3.5	5.7±4.9	3.2±1.9	0.24	−0.30

The principal point to be made here is that the variation in yields of recombinants from tube to tube is not exceptional. In this connection it must be mentioned that 5 of the 49 tubes contained *h* and *r* virus without any recombinants. Of these, one was exceptional in containing only 24 viral particles, and another for the extreme disproportion of parental types (88 percent *h*). The remaining three, each containing from 450 to 570 particles, are probably superimposed unmixed *h* and *r* bursts.

It should be noted also that the correlation between the yields of the two recombinants in this set is not significant, since the observed correlation is exaggerated by the tubes containing unmixed *h* and *r* bursts without recombinants. Whether the poor correlation is accidental, or an effect of the low multiplicity of infection, remains to be determined. The negative correlation between burst size and proportion of recombinants can, however, be ascribed to the superimposed mixed and unmixed bursts, as well as to the unmixed *h* and *r* bursts, in some of the tubes.

IDENTITY OF RECOMBINANTS WITH THE CORRESPONDING ANCESTRAL TYPES

According to any simple hypothesis of factorial recombination, one expects the recombinant virus arising in crosses not to differ genetically from the corresponding ancestral type. Two kinds of test indicate that this is so. In the first kind of test (HERSHEY and ROTMAN 1948) stocks of the phenotypic wild type arising from the cross between two different *r* mutants were backcrossed to authentic wild type. No *r* mutants appeared in such crosses, and it was concluded that the stocks were genetically identical.

The second kind of test is the following. The double mutant *h r7*, itself obtained by crossing the two single mutants, was crossed with wild type and the recombinants *h* and *r* were re-isolated. These were then tested by making the homologous (parental *h* by recombinant *h* and parental *r* by recombinant *r*) and heterologous (parental *h* by recombinant *r* and parental *r* by recombinant *h*) back crosses. In both cases, the homologous cross yielded only one type of

virus, and the heterologous cross yielded recombinants in the same proportion as found with the parental stocks. These tests show not only that the recombinants contain the same genetic markers as the corresponding progenitive types, but also that the region between the markers is unchanged.

MIXED YIELDS CONTAINING ONLY ONE PARENTAL TYPE OF VIRUS

Only four mixed bursts lacking one of the parental types of virus were found among the experiments reported in this paper. One, from the cross $h \times r1$, contained 86 percent h^+r and 14 percent $h\ r$. A second, from the cross $h \times r7$ contained 99.6 percent $h\ r^+$ and 0.4 per cent $h\ r$. A third, from the same cross with low multiplicity of infection, contained 83 percent $h\ r^+$, and 17 percent $h\ r$. The fourth, from the cross $h\ r7 \times$ wild type, contained 23 percent $h\ r^+$ and 77 percent $h\ r$. In this case the yield of $h\ r^+$, which appeared to be homogenous, formed atypical plaques and proved on isolation to differ from any known mutant of *T2H*. It seems reasonable to suppose that these exceptional bursts contained progeny stemming from mutants contaminating the parental stocks of virus. On the other hand, the one exceptional burst following low multiplicity of infection, together with the failure to find similar bursts among the crosses involving *h* and *r13*, suggest that a different interpretation should be looked for. Genetic tests which might have clarified this point are lacking. For the present we conclude, as a first approximation, that recombinants arise only in those bacteria in which both parental types of virus succeed in multiplying.

It may be added here, because the question arises in connection with these exceptional bursts, that no correlation can be seen between the proportion in mixed bursts of the total virus containing the *h* allele, and the proportion of the recombinant virus containing the *h* allele. We have therefore omitted this datum from the tables.

DISCUSSION

In collecting and analyzing the data just described, we have had in mind the following questions. Does genetic exchange occur in the course of matings between viral particles, or is it the expression of a mechanism of growth such as that visualized by LURIA (1947), according to which the multiplying units in the cell are not phage particles, but simpler structures derived from them? Can the linkage relations represented in fig. 1 be interpreted in terms of linear chromosome-like structures? Are the genetic exchanges reciprocal, as one expects for simple cases of crossing over, or must one look for an alternative mechanism more intimately connected with the mode of reproduction of the virus?

It was soon apparent that the data for crosses between linked and unlinked factors tended to give different answers to these questions, and we were led to consider a model based on two distinct mechanisms of exchange. The necessity for this arises from the following facts.

First, the linkage data indicate a limitation at about seven percent to the proportion of wild type found in crosses between linked factors, which is dif-

ficult to reconcile, in terms of a single mechanism, with the existence of a second class of crosses yielding about 15 percent of wild type.

Second, the correlation between proportions of the two recombinants in the cross *h*×*r7*, and the lack of a corresponding correlation in the cross *h*×*r1*, is incompatible with a single mechanism for the two crosses. It must be recalled, however, that the correlations found are too weak to be wholly convincing.

Third, LURIA's (1947) evidence for a mechanism of independent multiplication and transfer of subunits of the virus, and ours for a system of linkage, require dissimilar types of interpretation.

The model to which these considerations seem to lead is described below, but we do not consider that we have decisive answers to any of the questions originally posed. The remainder of this discussion is of value only insofar as it clarifies the questions, and systematizes the experimental results so far obtained.

The two linkage structures bearing the markers *r1* and *h*, respectively, are assumed to be examples of the class of independently multiplying subunits of the virus whose minimal number LURIA and DULBECCO (1949) estimate at about 25. The reconstitution of virus from these units must be regulated in such a way that each particle receives one representative of each kind of unit. In the cross *h*×*r1* the choice between *h* and h^+, and between *r* and r^+, is decided nearly at random to yield on the average 37 percent of recombinants and 63 percent of the parental types in bacteria yielding equal numbers of the two parents. The deficit of recombinants below 50 per cent is unexplained, but may be thought of as an effect of incomplete mixing between neighboring clones of multiplying virus in the cell.

According to this hypothesis one expects from the cross *h*×*r1* proportionate yields of the two recombinants in a single burst to be:

$$\mathrm{p}(h^+r^+) = \mathrm{m}\left(\frac{\mathrm{n}(h^+)}{\mathrm{n}(h^+) + \mathrm{n}(h)}\right)\left(\frac{\mathrm{n}(r^+)}{\mathrm{n}(r^+) + \mathrm{n}(r)}\right) \tag{4}$$

$$\mathrm{p}(hr) = \mathrm{m}\left(\frac{\mathrm{n}(h)}{\mathrm{n}(h^+) + \mathrm{n}(h)}\right)\left(\frac{\mathrm{n}(r)}{\mathrm{n}(r^+) + \mathrm{n}(r)}\right) \tag{5}$$

where the expressions on the left refer to proportions of recombinant virus, the corresponding expressions on the right refer to the intra cellular yields of the respective unit linkage structures, and the coefficient m expresses the fraction of the intracellular virus which may be regarded as a random mixture of the two parental types, the remainder being considered unmixed. If the structures carrying the markers *h*, h^+, *r*, and r^+ grow independently in the cell, their yields will fluctuate independently, and no correlation will be expected between the numbers of the two recombinants in sufficiently small yields of virus. This expectation is borne out by the data for viral yields from single bacteria. The average yields of the two recombinants are equal, however, showing that the several unit structures grow at equal rates. According to equations (4) and (5), the proportionate yield of recombinants should not be

influenced by burst size unless the latter has an effect on the fraction m. According to the data it does not, and m is a parameter having the average value 37/50. This interpretation requires that 26 percent of either kind of parental virus in the cell should multiply in effective isolation from the other parent.

It will be noticed that the expressions (4) and (5) reduce to mk if one makes the approximations mentioned in connection with equation (3). This provides a theoretical basis for the correction we have applied to yields of recombinants in crosses between unlinked factors. An analogous justification for its use in crosses between linked factors will appear in the discussion to follow.

The predictions for the cross *h*×*r7* are different from the preceding case, because here the markers are situated on homologous linkage structures, so that recombination requires something like crossing over, which in turn requires something like synapsis. The expected fractional yields of recombinants are

$$p(h^+r^+) = p(hr) = \tfrac{1}{2}\,msc, \qquad (6)$$

in which m has been defined previously, s is a fraction independent of m expressing frequency of pairing, and c is a crossover frequency. In order to make c independent of m and s it is evidently sufficient to define the product ms as the fraction of the viral yield made up of particles in which the marked unit has descended from an unlike synapsed pair. The application of (6) leads to ambiguity if exchanges can occur between descendants of unlike synapsed pairs (HERSHEY and ROTMAN 1948). In what follows this difficulty does not appear to be very serious, but no rigorous analysis has yet been attempted.

For the cross *h*×*r7*, if exchanges are reciprocal, one expects the correlation between proportions of the two recombinants to be disturbed only by fluctuations in relative growth of the two exchange products, in contrast to the cross *h*×*r1*, where the correlation is subject to fluctuations in the growth of four independent units. That these fluctuations are individually considerable is shown by the variations in relative and total viral yields in mixedly infected bacteria. A weak but probably significant correlation between proportions of the two recombinants is nevertheless visible in the cross *h*×*r7*. No such correlation can be seen in the cross *h*×*r1*. If the mechanism of exchange for these two crosses were the same, the greater correlation would be expected in the cross *h*×*r1*, which gives the larger yield of recombinants.

The predictions for the cross *h*×*r13* are the same as for *h*×*r7* except as modified by the much smaller yield of recombinants, presumably owing to a smaller frequency of crossing over. We have shown that in this cross the recombinants come from two or three individual exchanges per bacterium, and that there is little growth of recombinants subsequent to exchange. These circumstances ought to be favorable for testing the hypothesis of reciprocal exchange. The data are nevertheless inconclusive of this point.

The experiments provide information about the sequence of events in the cell. A mechanism of exchange limited to an initial phase of multiplication is ruled out by the following consideration. If exchange occurred at a time when there were few replicas in the cell, any cross yielding a small average number of

recombinants would show some individual bursts containing no recombinants and others containing a very large proportion, especially at low multiplicity of infection. Instead one finds a comparatively uniform yield of recombinants, and the distribution of their proportions is not affected by the multiplicity of infection.

Also if exchanges occurred freely throughout the period of multiplication of the virus, one would expect considerably greater variations in yields of recombinants than we have found. For instance, in the cross *h*×*r13*, in which there are only two or three exchanges per bacterium, the variations in yields of recombinants are not much greater than those expected to result from a random variation in the number of exchanges alone. Moreover, most of the bacteria yield only a few recombinants, so that little growth can have occurred subsequent to exchange. The conclusion is unavoidable that the exchanges are limited to the terminal phase of multiplication, or at any rate that recombinants are prevented from multiplying appreciably in most of the bacteria.

It is remarkable that the variations in proportions of recombinants are so little dependent on the degree of linkage (as between *h*×*r7* and *h*×*r13*), or on the postulated mechanism of exchange (as between the above and *h*×*r1*). The coefficients of variation in proportions of individual recombinants among single bursts are, for *h*×*r1*, about 40 percent; for *h*×*r7*, about 60 percent; and for *h*×*r13*, about 100 percent. This circumstance also supports the inference that the exchanges are limited to a late phase of multiplication.

The hypothesis stated permits one to examine further the structure of the linkage units. Since crosses between *r13* and any of the mutants belonging to the group closely linked to *r7* yield about the same proportion, seven percent, of wild type (HERSHEY and ROTMAN 1948), it might be supposed that one crossover between the distant markers is always accompanied by several others, so that 50 percent of the progeny of synapsed pairs of the units *r7* and *r13*, for example, would be recombinant types. If this supposition is correct the terms of equation (6) can be evaluated by setting $c = 0.5$ and $p(h^+r^+) = 0.07$. This gives 0.28 for the average fraction ms of virus descending from unlike synapsed pairs. If this fraction is assumed to be the same in other crosses involving the same linkage structure (the cross *h r7*×*r13* reported in this paper suggests that it is), one can write for them

$$p \text{ (wild type)} = 0.14\ c, \tag{7}$$

where c is the appropriate crossover frequency and the proportion of wild type is experimentally measured. The data for the three point crosses involving *r2*, *r3*, and *r6* (HERSHEY and ROTMAN 1948) are examined from this point of view in table 9. The proportions of wild type have been calculated for random crossing over between unit linear structures, using the crossover frequencies given by (7). It will be seen that the data are entirely compatible with the hypothesis tested. Additional tests of this kind are needed, however.

It will have been noticed that the average yield of recombinants in crosses between distant linked factors is very nearly half that found for unlinked factors. DR. M. DELBRÜCK has pointed out to us that this relationship can be

understood in terms of random pairing between homologous structures. In the simplest case one visualizes unrepeated pairing, that is, pairing limited to a phase in which there is no multiplication, and during which no structure finds more than one partner. For this case, the frequency of synapsis in equation (6) is simply the ratio of the number of unlike homologous pairs to the total number of homologous pairs. This ratio can be written

$$s = \frac{2ab}{(a + b)(a + b - 1)} \tag{8}$$

where a and b are the respective numbers of the two unlike homologous structures. Inspection of (8) shows that this ratio is essentially 2k as given by equation (3) when a+b is large compared to unity. Instead of equation (6) we have, therefore,

$$p \text{ (wild type)} = mkc, \tag{9}$$

from which the proportion of either recombinant expected in crosses between distant linked factors can be computed as follows.

The parameter m, taken to be the average fraction of virus randomly mixed in the cells at the time of reconstitution of virus from subunits, was found to

TABLE 9

Three Point Linkage Tests of Linear Structure

The symbol *r2,3* refers to the double mutant containing *r* alleles at the loci *r2* and *r3*, etc.
c_1 is the crossover frequency for the region between *r2* and *r3*.
c_2 is the crossover frequency for the region between *r3* and *r6*.
The locus *r3* is assumed to lie between *r2* and *r6*.
The factor 0.14 is explained in the discussion.

CROSS	FRACTIONAL YIELD OF WILD TYPE		COMPUTATION
	EXPECTED	FOUND	
r2×*r3*	—	0.020	$c_1=0.020/0.14=0.14$
r3×*r6*	—	0.014	$c_2=0.014/0.14=0.10$
r2×*r6*	0.030	0.024	$0.14[c_1(1-c_2)+c_2(1-c_1)]$
r2,3×*r6*	0.012	0.008	$0.14\ c_2(1-c_1)$
r3,6×*r2*	0.018	0.014	$0.14\ c_1(1-c_2)$
r2,6×*r3*	0.002	0.003	$0.14\ c_1c_2$

be 37/50 in crosses between unlinked factors. In (9) we require the corresponding fraction at the time of pairing, and assume this to be the same. The average of k for bacteria giving mixed viral yields (table 4) is 0.21, or 0.19 if one includes the ten percent of bacteria yielding only one type of virus. If k and m vary independently, their mean product is the same as the product of means, or 0.14 averaged over all bacteria. The average yield of either recombinant from equation (9), for factors sufficiently far apart so that c=0.5, is accordingly seven percent, computed solely from the data for crosses between unlinked factors. This is the maximum actually found in crosses

between linked factors (HERSHEY and ROTMAN 1948). Equation (9) also predicts, in agreement with the data for single bursts, proportionality between yields of recombinants and k.

The agreement supports the inferences previously drawn that the markers *r7* and *r13* are attached to the same linkage unit, and that the frequency of crossing over between them is 0.5. It suggests that the pairing itself is complete without appreciable repetition, and occurs at random except that about 26 percent of the units of each kind are effectively segregated from their opposite numbers. The measure of this segregation, m, is on this view the same for crosses between linked and unlinked factors. On the other hand, this interpretation cannot be rigorously correct, because one can show by multiple factor crosses (HERSHEY and ROTMAN 1948) that repeated exchanges, or exchanges among three viral particles, occur. An estimate of the amount of repeated pairing has not yet been attempted, except that the considerations just offered suggest either that it is small, or that random pairing is limited to a small proportion of the population.

It follows from equation (9) that the interpretation in terms of orderly pairing accords with the fact, otherwise very puzzling, that the proportion of recombinants is not affected by size of burst even in crosses between linked factors.

It has been seen that the linkage data support fairly well the idea of linear structure, but independent evidence for crossing over is meagre. According to any simple model of reciprocal exchange, a correlation between proportions of sister recombinants in individual bursts would be expected. This expectation has been only partially realized, and the question arises whether the linkage data themselves require the crossover hypothesis. The following model, suggested by DR. A. H. STURTEVANT, shows that they do not, and also shows that the question of reciprocity is closely connected with the question whether the exchanges are material transfers.

Suppose that the replication of linear structures occurs zipperwise along the pattern from one end to the other, but that the partners separate prematurely to yield fragmentary replicas. Additions to the fragments are subsequently possible only after pairing with the same or another homologous structure, which in mixedly infected bacteria could belong either to the same or a different parental line. Genetic recombination in a two factor cross will depend, then, on the contingency that the two marked regions of a given replica be laid down one after the other on homologous structures from the two unlike parents. With simple assumptions, all the consequences of the crossover hypothesis (equation (6)) follow from this model, except that the independent origin of the two recombinants provides an additional source of independent variation in their numbers.

The complications peculiar to this model have to do principally with the evidence that exchanges occur only during the terminal phase of growth. These complications are not very serious if one assumes that during early stages of growth the probability is great that a fragment will be started and completed on patterns belonging to the same parental line; that is, that the

mixing of the cell contents is relatively incomplete, and the distance between unlike clones relatively great, for small total populations. It has to be stipulated further that the terminal mixing is independent of the final concentration of virus in the cell, to account for the lack of dependence of proportion of recombinants on burst size. Some hypothesis of this sort may prove useful if further experiments fail to strengthen the present evidence for reciprocal exchange.

It is notable that two very different lines of evidence, ours and that of LURIA (1947), have led to the idea of independently multiplying subunits of the virus. Our results differ from LURIA's only in calling for a system of linkage superimposed on the set of independent units. It remains to be seen whether a combination of genetic and radiological techniques bears out the present conclusions, and perhaps leads to an identification of the radiation-sensitive units with the linkage structures.

SUMMARY

Genetic recombination between two viruses differing by two mutational steps has been studied by infecting bacteria with the pair, and counting the numbers of the four types of virus found in yields from single bacteria. The crosses so examined include *h*×*r1* (unlinked), *h*×*r7* (linked), and *h*×*r13* (closely linked), where *h* refers to a mutant of altered host range, and *r1*, *r7*, and *r13* are different mutations producing the same alteration in type ol plaque. The reverse crosses, *h r*×wild type, were also studied. The results may be summarized as follows.

Nearly all mixedly infected bacteria yield both parental types of virus and two recombinants, according to the scheme *h*+*r*=*h r*+wild type. The ten percent or so of bacteria yielding only one of the parental types seldom or never yield any recombinants. The rest of the bacteria always yield two recombinants, except for the occasional absence of one or both in the crosses between closely linked factors.

The average yields of the two recombinants in any one cross are the same, and are independent of the direction of exchange, so that reverse crosses involving the same pair of mutant factors yield the same number of recombinants. The proportionate yields of recombinants from individual bacteria are independent of burst size, and of the total multiplicity of infection, but depend on the relative yields of the two parental types. The effect of the latter is not marked, however, and the variations from bacterium to bacterium must be chiefly the result of variations in the number of genetic exchanges and in the growth of recombinants subsequent to exchange. These variations may be described by saying that one finds a moderately skewed distribution, with mode less than the mean, and with mean and standard deviation dependent on the linkage relations as follows: for *h*×*r1*, 15 ± 6, for *h*×*r7*, 7 ± 4, for *h*×*r13*, 1 ± 1, expressed in round numbers as percent of either recombinant in the total yield of virus.

A weak but moderately convincing correlation between the proportionate

yields of the two recombinants in individual bacteria is discernible in the cross *h*×*r7* and its reverse, but not in the other crosses.

In the cross *h*×*r13* only two or three genetic exchanges occur during the multiplication of the virus in a single bacterial cell. These exchanges take place near the end of the period of multiplication of the virus.

A hypothesis is outlined which is compatible with the genetic data and with the results of LURIA concerning reactivation of irradiated virus in bacteria receiving two or more individually noninfective particles. The hypothesis is an extension of that of LURIA, according to which one visualizes genetic interaction not between two viral particles, but between two sets of independently multiplying chromosome-like structures. Genetic exchange occurs either by reassortment of these structures, or by something like crossing over between homologous pairs, depending on the structural relation between the genetic factors concerned. The interpretation made brings the linkage relations into superficial agreement with the requirements of linear structure, but there is little evidence that the genetic exchanges are reciprocal, and accordingly little evidence that they are material exchanges.

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MUTATIONS DURING THE GROWTH OF BIOCHEMICAL MUTANTS OF ESCHERICHIA COLI[1]

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MICROORGANISMS of all sorts are renowned for their ability to adjust satisfactorily to unfavorable changes in their environment. This phenomenon is called adaptation. When a microorganism possesses a growth factor requirement, the establishment of a culture in the presence of a limiting amount of the required substance will frequently involve the development of the lost synthetic ability. Each culture is, of course, a population of large numbers of organisms and adaptation must be analyzed in these terms. In certain instances, where this has been done, adaptation could be accounted for by mutation and selection (RYAN 1946).

Considerable attention has been paid in our laboratory to a mutant of *Escherichia coli* which is deficient in its ability to synthesize histidine and requires the addition of the amino acid to the medium. When a culture of this mutant, grown in the presence of histidine, is washed and inoculated into medium containing histidine, growth is complete within twelve hours. A similar inoculum in medium devoid of histidine exhibits no growth during the first twelve hours but invariably grows within twenty four hours. On the other hand, when some of these adapted bacteria are washed and inoculated into fresh medium devoid of histidine growth does occur within twelve hours. The adapted organisms thus behave in the absence of histidine as the parent culture does in its presence. The adapted strain is histidine-independent and this character is inherited indefinitely.

This paper will deal with the mutations—the occurrence of those changes which result in new synthetic activities. The adaptive consequences of these changes are the subject of other communications (RYAN 1948, RYAN and SCHNEIDER 1948).

MATERIALS AND TECHNIQUES

All of the biochemical mutants of *Escherichia coli* which we have studied were secured after X-ray treatment. The following strains were generously supplied for our studies by DRS. J. O. LAMPEN and R. R. ROEPKE who obtained them from wild-type strain No. 15: histidineless (148-334, 161-489, 225-484, 654-462), lysineless (15L-171), arginineless (1572-228), methionineless (532-171), prolineless (209-301), tryptophaneless (1250-228) and threonineless (558-228) (ROEPKE, LIBBY, and SMALL 1944). Strain 697–680 which is both leucineless and threonineless was kindly supplied by DR. E. L. TATUM

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who secured it from a wild-type strain K-12 (TATUM 1944). The bulk of our observations were made on strain 148-334 and, unless otherwise mentioned, the term "histidineless" will refer to these organisms.

The methods used in maintaining stocks, inoculating cultures, and measuring growth are described elsewhere (RYAN and SCHEIDER 1948). The "chemically defined" minimal medium had the following composition: NH_4Cl, 5 g; NH_4NO_3, 1 g; Na_2SO_4, 2 g; $MgSO_4.7H_2O$, 0.1 g; K_2HPO_4, 3 g; KH_2PO_4, 1 g; $CaCl_2$, ca. 1 mg; trace elements, 1 ml (RYAN, BEADLE and TATUM 1943); Bacto-asparagine, 1.5 g; glucose, 5 g; H_2O, 1 liter. Bacto-agar before use was scrupulously washed fifteen times with double volumes of distilled water and twice with alcohol. This procedure removes not only inhibitors but also growth factors. Histidine, the substance most commonly studied, was supplied as Eastman L-histidine monohydrochloride. H_2O. Medium "with histidine" will, in this paper, refer to a medium containing 25γ per ml of the Eastman product mentioned.

Before plating or inoculation cultures were washed by centrifuging and resuspending twice in 0.9 percent sodium chloride. This solution was also used for dilutions. Each Petri dish was first supplied with a bottom layer of from 5–10 ml of 2 percent agar in minimal medium which was allowed to harden. Onto the surface of this was introduced the appropriate bacterial suspension which was then mixed with another 10 ml of minimal agar. Finally, after this had set another 5–10 ml of minimal agar was layered on top. Unless otherwise mentioned the plates were incubated at 37°C for two days at which time the colonies formed were counted and their positions marked with glass marking ink on the bottom of the dishes. At this time the plates may again be layered with agar (or liquid) containing enough histidine to bring, after diffusion is complete, all of the agar to a concentration of 25γ per ml. Such plates were incubated for another two days at 37°C and the newly formed colonies counted.

RESULTS AND CONCLUSIONS

The adaptive growth of the histidineless (h^-) culture in the absence of histidine is the result of the action of histidine-independent (h^+)mutants. When an h^- culture purified by repeated colony isolations, is grown in the presence of histidine, washed and plated into minimal agar a small number of colonies appear. These colonies upon isolation, purification and test appear to consist of h^+ organisms which are stable with regard to their ability to synthesize their own histidine.[2] The h^- organisms on the plate form similarly large and visible colonies only when supplied with histidine by layering. By such a procedure the number of h^+ organisms produced during the growth of an h^- culture could be determined.

Before this procedure was used its reliability was investigated. In the first place determinations of the total number of bacteria in an h^- culture did not differ when the culture was plated directly into agar containing histidine or

[2] An examination, by the plating and layering technique, of more than 15,000 bacteria from an h^+ culture, grown in the presence of histidine, revealed no h^- organisms.

plated into minimal agar and subsequently layered. By the first method in two experiments 45 and 37×10^7 bacteria were found; by the second method in the same two experiments 49 and 41×10^7 were determined respectively. The h^- cells on the minimal plate do not die for reasonable periods of time but remain capable of forming an h^- colony when layered with histidine. In one experiment seven minimal agar plates containing h^- bacteria were prepared and every day another one of these was layered. The average number of colonies formed was 44 ± 3; there were 49 on the plate layered on the first day and 43 on the plate layered on the seventh. Similarly, in another experiment, where each plate contained, in addition, 64 ± 5 h^+ colonies, an average of 58 ± 7 h^- colonies developed after layering; 49 on the first plate and 62 on the plate layered after the ninth day. In order to ascertain whether the colonies that arise on the minimal agar plate consist of h^+ bacteria while those that come up after layering are h^- a number of each type were isolated and tested

TABLE 1

The number of h^+ colonies per ml from serial dilutions of different h^- cultures when plated on minimal agar.

DILUTION	CULTURE						
	1	2	3	4	5	6	7
10^0	441	185	88	78	325	58	59
10^{-1}	53	20	11	26	37	11	9
10^{-2}	11	5	5	7	10	5	5
10^{-3}	4	5	2	1	6	3	2
10^{-4}	7	3	7	4	7	2	5
10^{-5}	4	3	3	7	6	1	3
10^{-6}	1	2	1	2	1	0	0
10^{-7}	0	0	0	0	1	0	0
T†	50	44	32	37	59	39	34

† Total number of bacteria ($\times 10^7$) per ml determined by layering with histidine.

for hisitidineless character. Twenty-six colonies which appeared after layering with histidine were isolated into medium devoid of histidine. A loop of this suspension was immediately transferred into medium with histidine. After 12 hours, growth had occurred only in the histidine medium. Therefore the colonies consisted of h^- organisms. Thirty-two colonies isolated from minimal agar plates tested h^+. In this sample, then, the plating and layering technique seemed without error in characterizing the proportions of h^+ and h^- bacteria in a culture. However, further investigation revealed a number of anomalous situations.

The most representative of these was the disproportionately high frequency of h^+ colonies in low members of serial dilutions. Table 1 shows the number of h^+ colonies on minimal agar plates prepared from serial dilutions of h^- cultures grown in the presence of histidine. The number of h^+ colonies per plate does not decrease in accordance with the dilutions of the cultures that were plated

but rather calculations of the total number of h^+ organisms present in the original cultures are thousands of times greater when based on 10^{-5}, 10^{-6}, or 10^{-7} dilutions than when based upon platings of whole cultures. This phenomenon did not seem to bear any relation to the past histories of the inocula which varied in age from 18 to 31 hours and which were derived from stock tubes between two days and four months old. Furthermore, among the seven cultures shown in table 1 there are represented strains secured from four different single colony isolates. The appearance of colonies on plates from very dilute suspensions did not seem to be due to the carry-over of histidine from the culture medium because the inocula were washed. After two centrifugations and resuspensions in saline, there is not enough histidine per bacterium as determined by bioassay, to allow more than one division. Further, the h^+ colonies are discrete and located in agar unclouded by bacteria.

It might be supposed that the number of h^+ colonies on plates from low dilutions is the true index of the number of h^+ bacteria in the original culture because of inhibitions on more crowded plates. On plates from 10^{-6} dilutions there were several hundred h^- bacteria, and on plates from undiluted cultures there were about half a billion. On this hypothesis various attempts were made to secure correction factors which could be used to compensate for the inhibition and equate the ratios of h^+ colonies to h^- organisms on plates from the different dilutions. These attempts were without success because factors calculated from one experiment did not apply to a second. It was concluded that something was operating which varied from experiment to experiment and influenced the number of colonies formed upon plating the various dilutions.

As a tentative explanation it could be assumed that there are two types of h^+ organisms, one sensitive to the crowding action of h^- bacteria and the other resistant. The colonies on plates from 10^0 dilutions consist of resistant h^+ bacteria; the sensitive h^+ bacteria also present are inhibited and do not form colonies. The most sensitive bacteria form colonies only on platings of 10^{-6} or 10^{-7} dilutions. Mutations to resistance would account for the large variance observed among plates from different whole cultures. It remains another matter to account for the small variance observed after plating 10^{-6} dilutions. This hypothesis was tested by isolating several h^+ colonies suspected of being resistant (from 10^0 plates) and several thought to be sensitive (from 10^{-6} plates). These were tested in two ways. Several hundred h^+ bacteria were mixed with about 10^9 h^- bacteria and serial dilutions of the mixture were plated into minimal agar. The number of h^+ colonies introduced by the h^- culture, as determined by a control plating, was subtracted from the total number of h^+ colonies per plate leaving the number of h^+ colonies formed by the h^+ bacteria which were deliberately introduced. The resistant h^+ bacteria were expected to form colonies whose number varied in accordance with the dilution, while it was presumed that sensitive h^+ bacteria would not form colonies in numbers equal to those introduced. In actuality, not only did the resistant bacteria yield colonies whose numbers decreased tenfold with tenth dilutions but so did the bacteria suspected to be sensitive. Similarly when

about 50 "sensitive" or "resistant" h^+ bacteria were mixed with about 5×10^8 h^- bacteria and plated without dilution into minimal agar it was impossible to distinguish the two types of h^+ organisms on the basis of the efficiency with which they produced h^+ colonies. Table 2 shows the relationship of the total numbers of h^+ colonies found per plate to the number expected on the basis of the h^+ organisms introduced from the h^+ culture and from the h^- culture. Neither type of h^+ bacteria was inhibited by the crowding action of the h^-

TABLE 2

The number of h^+ colonies formed on minimal agar plates from suspensions of h^+ bacteria, h^- bacteria and a mixture of the two. Each number is the average of counts of three plates each from two experiments.

STRAIN OF h^+	NUMBER OF h^+ COLONIES FROM h^+ STRAIN ALONE	NUMBER OF h^+ COLONIES FROM h^- STRAIN ALONE	NUMBER OF h^+ COLONIES EXPECTED FROM MIXTURE OF h^+ AND h^- STRAINS	NUMBER OF h^+ COLONIES FOUND FROM MIXTURE OF h^+ AND h^- STRAINS
Resistant 1	57	96	153	150
Resistant 2	55	101	156	155
Sensitive 1	68	84	152	158
Sensitive 2	45	84	129	132
Sensitive 3	28	90	118	138
Sensitive 4	45	90	134	126

organisms and as a consequence the hypothesis of sensitivity and resistance was discarded.

Indeed, it seemed reasonable to question whether the plating technique yielded a reliable characterization of the number of h^+ organisms in a culture. From table 1 it can be seen that there are about 5×10^8 h^- organisms per ml of h^- culture, and, assuming that the plating technique yields valid information, about 1×10^6 h^+ organisms. Consequently, one ml samples of a 10^{-9} dilution of an h^- culture should rarely contain an h^+ organism but should possess, on the average, 0.5 h^- bacteria. Such 10^{-9} dilutions of washed h^- cultures were prepared and one ml samples were inoculated into 10 ml lots of liquid medium with and without added histidine. Some of the cultures containing histidine grew because the one ml samples with which they were inoculated contained one or more bacteria. The remainder failed to grow because they received no bacteria in the one ml sample. If the distribution of bacteria in the one ml samples was due to chance it should follow the Poisson distribution and conversely this formulation ($p\,(0) = (m^0/0!)e^{-m}$) allows the average number of bacteria in the samples to be calculated from the frequency of cultures that fail to grow. Table 3, which contains results averaged from three experiments, shows that the average inoculum size so calculated is the same as that obtained by plating the inoculum in the presence of histidine.

This table shows, in addition, that the inocula introduced into minimal medium grew in approximately the same percentage of cases as when introduced into medium with histidine (even if this occurred after 36 instead of 24 hours). This result is in strong contrast to the plating data, which would indicate that there are no h^+ organisms in one ml of a 10^{-9} dilution of an h^- culture. Apparently every h^- organism is capable of yielding a growing culture even in the absence of added histidine. In confirmation of this is the fact that 100 one ml samples of a 10^{-7} dilution of an h^- culture all yielded growth in minimal medium. Of 45 one ml samples of a 10^{-8} dilution only 4.4 percent

TABLE 3

The inoculation of one ml samples of a 10^{-9} dilution of an h^- culture into a number of tubes with and without added histidine.

	WITH HISTIDINE	WITHOUT HISTIDINE
Number of cultures	140	132
Number that failed to grow	83	72
Percent that failed to grow	59	55
Calculated average inoculum size	0.53	0.60
Observed number of h^+ bacteria*	0	0
Observed number of h^- bacteria*	0.51	0.51

* In inoculum plated as 1 ml of a 10^{-7} dilution.

failed to grow, a fraction statistically no different from the 2.5 percent that failed to grow when inoculated into medium containing histidine. The growth that occurs in minimal medium consists, as determined by plating, almost entirely of h^+ organisms. Consequently, the h^+ organisms must have arisen by mutation from bacteria that, as characterized by plating, were h^-.

This raises the important question whether mutations from h^- to h^+ are so frequent in the absence of histidine as to occur in every bacterium. Or were the single h^- bacteria able to reproduce sufficient h^- offsprings in minimal medium to make a mutation occurring with orthodox frequency a probable event? A decision on this point required examining, after short periods of time, liquid cultures into which single h$^-$ bacteria had been introduced. After 12 hours such cultures were plated and shown to contain about 10^5 h^- bacteria. Despite the fact that no histidine had been added as such to the medium about 17 generations of bacteria had been produced from what, on the plating criterion, had been an h$^-$ organism. Once it had been demonstrated that h^- bacteria continue to grow in liquid minimal medium, their behavior on agar was more exactly examined. The h^+ colonies which appear when h^- cultures are plated into minimal agar are clearly defined lenses about 2 mm in diameter. The agar in which they reside is perfectly clear although many h^- bacteria may have been mixed with it. When such agar, however, is examined at a magnification of about 100 times, small colonies, not visible to the naked eye, are apparent. Each of these microcolonies was formed from an h^- bacterium

and when the plate is layered with agar containing histidine it is these microcolonies which enlarge to form the visible h^- colonies. Each microcolony contains about 10^4 bacteria while a colony visible to the naked eye contains more than 10^7. Since the h^- organisms are able to grow either in minimal medium or on minimal agar it is necessary to account for the fact that each h^- bacterium does not form an h^+ colony on agar, while in liquid medium, it forms an adapted culture consisting of h^+ organisms.

The growth of a colony in agar is restricted and the bacteria are closely crowded. It might be expected, since it is known that h^- organisms can prevent the growth of h^+ (RYAN and SCHNEIDER 1948), that in a microcolony some mutations from h^- to h^+ do occur but only fortuitous h^+ mutants are able to excape the influence of their h^- parents, to reproduce and to form an h^+ colony. In order to discriminate between this hypothesis and one which

TABLE 4

Growth produced by inoculating h^- bacteria in the absence of added histidine into semi-solid agar and liquid medium kept under stationary and shaken conditions.

		TURBIDITY
Shaken	Liquid medium	495
	Semi-solid agar	416
Unshaken	Liquid medium	132
	Semi-solid agar	17

would place on the agar itself the responsibility for the difference from results in liquid, an experiment was constructed which made use of semi-solid minimal agar. This could be allowed to remain stationary, in which events its rigidity kept the daughter bacteria crowded together, or it could be constantly shaken so that contained colonies were completely dispersed and crowding was reduced to a minimum. The number of h^- bacteria which were introduced into the semi-solid agar, which was contained in a flask, was determined by plating a similar sample of the inoculum in 2 percent agar which was later layered with histidine. Similar samples of the inoculum were also introduced into liquid medium, in one case allowed to remain stationary in the flask bottom, in another case constantly shaken with one of the flasks containing semi-solid agar. This experiment was performed three times and each time each condition was run in triplicate. The averaged results are shown in table 4. An average of five h^- bacteria was introduced into each of the flasks. The amount of growth achieved under shaken conditions on liquid medium is approximately equal to that on semi-solid agar. Cultures grown under both of these conditions consisted almost exclusively of h^+ bacteria. The same was true of stationary liquid cultures although the amount of growth was much reduced. Nevertheless it was considerably more than that exhibited by stationary semi-solid agar

cultures. The average turbidity of 17 is based upon those three semi-solid agar cultures which exhibited no colonies. When plated out these cultures were shown to contain about 20×10^6 h^- and only about 10 h^+ organisms per ml. Nevertheless these h^+ bacteria did not form visible h^+ colonies. In the remaining stationary semi-solid agar cultures the h^+ organisms did form visible colonies. However, when these colonies were dispersed in the agar they exhibited turbidities ranging from 37 to 121. In no case was the amount of growth equal to that in stationary liquid cultures where crowding was reduced by the free movement of the bacteria. It may be concluded from these experiments that, although h^- organisms reproduce and form colonies on minimal

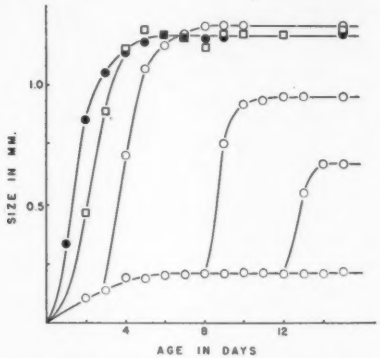

FIGURE 1.—The increase in size of colonies from an h^- culture diluted to 10^{-7} and plated on minimal agar. There was an average of 61 bacteria per plate. The plates were kept at 37°C in moist chambers. The lower curve, passing through open circles, describes the average size of between 9 to 25 h^- microcolonies. The ascending curves, also passing through open circles, describe the further growth of individual h^- microcolonies when the plates containing them were layered with agar containing histidine at 3, 8 and 12 days. The open squares describe the adaptation of an h^+ colony which arose from an h^- bacterium. The black circles describe the growth of an h^+ colony formed after plating a 10^{-7} dilution of an h^+ culture in the absence of histidine. On these plates there was an average of 23 bacteria.

agar, the h^+ bacteria produced by mutation may not always be able to escape the inhibition of crowding to form h^+ colonies. In liquid medium, however, these h^+ mutants will form adapted cultures.

The inhibition of h^+ bacteria by h^- may be brought about by factors originating and operating within a colony or the factors may operate between colonies. Furthermore, the inhibition is not restricted to h^+ bacteria but limitations on the growth of h^- organisms may also be imposed. These points were demonstrated by a study of the growth in time of h^- microcolonies on minimal agar plates. Figure 1 shows the increase in size of such colonies as determined with a micrometer ocular. It can be seen that the microcolonies achieve a maximum size within about four days and thereafter remain at constant size. When supplied with histidine these microcolonies rapidly develop into visible h^- colonies whose maximum size roughly depends upon the age of the plate

at layering. The h^- colonies when supplied with histidine grow at about the same rate as h^+ colonies from an h^+ culture. Similarly, h^+ colonies formed on plates of h$^-$ bacteria grow at the same rate. The size to which unadapted h^- microcolonies will grow before adaptation depends upon the total number present on a plate. Figure 2 shows the growth curves of h^- microcolonies under different conditions of crowding. The fewer microcolonies per plate the larger they will grow before assuming stationary size. Crowding does not prevent the growth of adapted h^+ colonies arising from the microcolonies but influences the size they will attain and, as will be shown below, determines the fre-

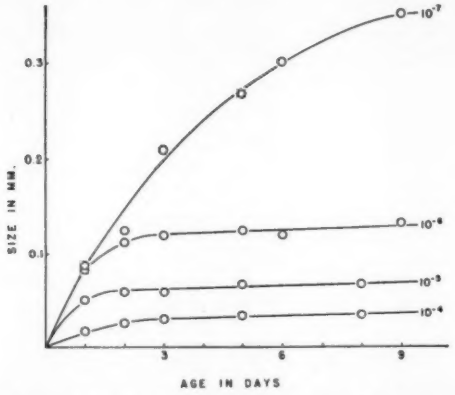

FIGURE 2.—The increase in size of h^- microcolonies arising on minimal agar after plating different dilutions of an h^- culture. These curves are each based upon measurements of from 9 to 25 microcolonies. The plates containing the 10^{-7} dilution possessed on the average 53 h^- microcolonies.

quency with which they appear. Figure 3 shows how the final size of h^- colonies grown in the presence of histidine is also a function of the total number of bacteria per plate.

The adapted h^+ colonies do not only arise during the early growth of the microcolonies but they may appear for some time after the microcolonies have achieved a stationary size. A count after a definite time, such as two days, of the number of h^+ colonies formed by plating an h^- culture is deceptive. Figure 4 shows how the number of h^+ colonies increases with time. For about ten days on plates containing 10^{-7} dilutions of an h^- culture the rate of increase is approximately linear. This increase occurs during the period that the h^- microcolonies on such plates are increasing in size (figure 2). In the presence of higher densities of microcolonies, however, the rate of increase is not linear but rapidly falls off so that the total number of h^+ colonies becomes constant. Figure 5 contains a series of curves showing the rate of increase in number of h^+ colonies on minimal agar plates and containing different dilutions of an h^- culture. These curves do not level off as soon as the h^- microcolonies cease

growing but somewhat thereafter. The most significant feature of these curves is that when they are extrapolated towards the ordinate not all intercept it in the positive region. When the number of h^+ colonies at the point of interception is determined they are found to decrease approximately ten-fold with

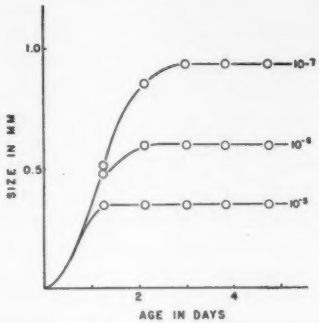

FIGURE 3.—The rate of growth of h^- colonies on agar covered with a second layer of histidine-containing agar immediately after plating. The points are the average measurements of two colonies. The plates made from the 10^{-7} dilution contained an average of 39 colonies.

the tenth dilutions. This coincidence suggests that the extrapolations may reveal the true number of h^+ organisms which were in the h^- culture at the time it was plated. If this were true, then extrapolations of this sort should yield values equal to a known number of h^+ organisms deliberately mixed with various dilutions of an h^- culture. Figure 6 shows a series of curves obtained from an experiment of this sort. It can be observed that the extrapolations of all of the curves pass through some positive value of the ordinate. These values are equal to the number of h^+ organisms introduced into each dilution plus the number which were already present. In those dilutions which, on the

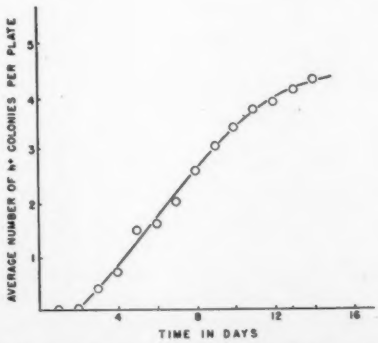

FIGURE 4.—The rate of increase in the number of h^+ colonies formed on minimal agar plates containing an average of 61 bacteria each. Each point is averaged from counts of 27 plates.

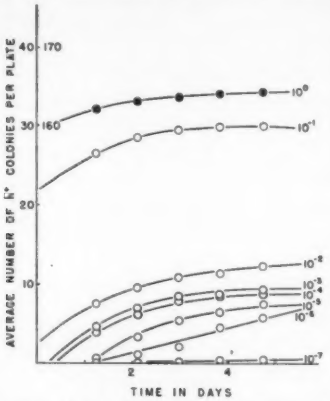

FIGURE 5.—The rate of increase in the number of h^+ colonies on minimal agar plates containing different dilutions of an h^- culture. The 10^{-7} dilutions contained, on the average, 35 bacteria per plate. Each point is the average of counts of 10 plates. The scale on the inside of the ordinate is for the undiluted culture; that on the outside for the remainder.

basis of the information in figure 5, were without any spontaneously occurring h^+ bacteria, the values on the ordinate through which the extrapolations pass should equal the number of deliberately introduced h^+ bacteria. Table 5 shows that this was true in two separate experiments. In one instance, when an average of 12 h^+ bacteria was added, extrapolations of the data from 10^{-3} to 10^{-7} dilutions passed through the ordinate at an average level of 13. In another instance when an average of five h^+ bacteria was added the extrapolations aver-

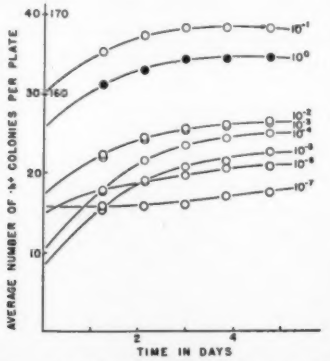

FIGURE 6.—The rate of increase in the number of h^+ colonies on minimal agar plates containing different dilutions of an h^- culture. To each plate had been added samples of a suspension of h^+ bacteria, which, as determined by separate plate counts contained about 12 h^+ bacteria. The plates containing the 10^{-7} dilutions possessed, on the average, 44 bacteria. Each point is the average of counts of 10 plates. The scale on the inside of the ordinate is for the undiluted culture; that on the outside for the remainder.

aged 4. Consequently, it may be concluded that determination of the number of h^+ colonies with time yields data which allows the calculation of the true number of h^+ organisms in the original culture. The precision of this method is fairly good. Although the extrapolations are drawn by eye the curves which can be drawn through the points do not strike the ordinate at very different levels. If whole h^- cultures are plated and the h^+ colonies counted from day to day, the error introduced by the extrapolation procedure is well within the error of sampling. Hence, the best characterization of the number of h^+ organisms in an h^- culture is obtained by a 10^0 plating with the possible refinement of determining the slight increase with time and extrapolating.

TABLE 5

The number of h^+ bacteria per ml of different dilutions of an h^- culture determined by extrapolating plate counts.

	DILUTION	h^+ BACTERIA ADDED		h^+ BACTERIA NOT ADDED
		UNCORRECTED	CORRECTED	
Experiment 1.	10^0	156	143	160
	10^{-1}	30	17	22
	10^{-2}	16	3	3
	10^{-3}	16	—	0
	10^{-4}	10	—	0
	10^{-5}	9 (13*)	—	0
	10^{-6}	15	—	0
	10^{-7}	15	—	0
Experiment 2.	10^0	111	107	118
	10^{-1}	13	9	11
	10^{-2}	5	1	0
	10^{-3}	4	—	0
	10^{-4}	4	—	0
	10^{-5}	3 (4†)	—	0
	10^{-6}	6	—	0
	10^{-7}	4	—	0

* Twelve h^+ bacteria, as determined by separate plate count, were added to each plate.
† Five h^+ bacteria, as determined by separate plate count, were added to each plate.

The problem of accounting for the formation of microcolonies by the h^- bacteria in the absence of histidine still remains to be considered. There are three reasonable hypotheses. The first is that the h^- bacteria inoculated into the plates carry over small amounts of histidine which are used for a slight amount of growth. Against this notion is the fact that the histidine content of the inoculum is not sufficient to account for the growth observed. Hydrolysates were prepared of washed h^+ cultures which had grown on minimal medium and of washed h^- cultures which had grown in the presence of optimal (25γ per ml) and limiting (0.8γ per ml) histidine. These were assayed for their histidine content by the use of the growth of h^- bacteria. The h^+ organisms

contained $3.0 \pm 0.4 \times 10^{-9}\gamma$ of histidine per bacterium; the h^- organisms from the culture with optimal histidine, $3.2 \pm 0.4 \times 10^{-9}\gamma$; and the h^- bacteria from the cultures with limiting histidine, $1.8 \pm 0.2 \times 10^{-9}\gamma$. The addition of known amounts of histidine to these hydrolysates allowed 94 percent recovery. Apparently there is some storage of histidine in the presence of a surplus, but only 25 percent of what was supplied to the limited cultures was recovered in the cells. Hence, the store is enough to allow for at the very most only one division per bacterium. It seems unlikely that the carry-over of histidine was responsible for microcolony formation.

It might be that the agar in the plates is contaminated with a small amount of histidine. However, every h^- bacterium was able to form a culture when inoculated into liquid medium to which no histidine had been added. Hence, agar is not necessary for the continued division of h^- bacteria in minimal medium. But is the liquid medium itself contaminated with histidine? This medium contains, in addition to the inorganic salts, two organic substances, glucose and asparagine. In our experience the role of asparagine has been to decrease the lag period slightly. Viability, the rate of growth during the logarithmic phase and the size of the final crop of bacteria are the same in the absence as in the presence of asparagine. When asparagine is omitted from the minimal medium used in plating, the results shown in table 6 are obtained. Perfectly orthodox tenth decreases in the number of h^+ colonies result from tenth dilutions of the h^- culture. The anomalous incidence of h^+ colonies in low dilutions can be brought about either by the addition of asparagine or of small amounts of histidine to the agar. In order to discover whether the action of asparagine was as such or due to the contaminating presence of small amounts of histidine, a sample of asparagine was passed through a column of Filtrol-Neutrol, a substance which is known to adsorb histidine (MARTIN and SYNGE 1945). After such treatment the asparagine was inactivated and required the addition of small amounts of histidine to allow colony mutation. The amounts of histidine required to mimic the effect of 0.15 per cent asparagine are extremely small and could not be determined by any available chemical or microbiological method. Our asparagine preparation is actually quite pure with regard to histidine, containing only about 0.003 percent. Yet this is sufficient to allow the growth necessary for microcolony formation and consequent mutation.

It is also sufficient to allow for the growth observed in liquid medium inoculated with single h^- bacteria. Table 7 shows the results of inoculating 1 ml samples of a 10^{-9} dilution of an h^- culture into liquid medium with and without asparagine and with and without histidine. When inoculated into medium containing asparagine every single bacterium grew even though no histidine had been added (see table 3). However, when inoculated into medium containing asparagine from which the histidine had been removed none of the bacteria grew unless histidine was added. Similarly, in medium devoid of asparagine and histidine only one of the single bacteria formed a culture. We believe, for the following reasons, that this single exception resulted from an error of some sort.

TABLE 6

The number of h^+ colonies from serial dilutions of an h^- culture when plated on agar containing different amounts of histidine and asparagine.

DILUTION	NO ASPARAGINE				NO ASPARAGINE PLUS 0.01γ HISTIDINE PER ML		NO ASPARAGINE PLUS 0.1γ HISTIDINE PER ML		0.15 PERCENT ASPARAGINE		0.15 PERCENT TREATED* ASPARAGINE		0.15 PERCENT TREATED* ASPARAGINE PLUS 0.01γ HISTIDINE PER ML	0.15 PERCENT TREATED* ASPARAGINE PLUS 0.1γ HISTIDINE PER ML
	EXPERIMENT													
	A	B	C	D	A	B	A	B	C	D	C	D	D	D
10^0	155	158	81	22	133	164	—	—	87	55	71	35	32	54
10^{-1}	15	22	11	3	20	26	33	43	13	14	16	7	2	21
10^{-2}	3	0	4	1	1	2	18	10	9	7	1	0	0	13
10^{-3}	1	0	0	0	2	0	23	14	5	6	0	1	2	3
10^{-4}	0	0	0	1	1	5	15	20	12	10	0	0	1	10
10^{-5}	0	0	0	0	2	1	24	23	9	2	0	0	1	17
10^{-6}	0	0	0	0	0	0	11	9	3	2	0	0	1	13
10^{-7}	0	0	0	0	0	1	1	1	2	1	0	0	0	21
T†	—	—	36	21	—	—	—	—	36	20	36	23	19	22

* The treated asparagine was passed through an adsorbent column of Filtrol-Neutrol which would be expected to remove the histidine.

† Total number of bacteria ($\times 10^7$) per ml determined by layering with histidine.

TABLE 7

The inoculation of one ml samples of a 10^{-6} dilution of an h^- culture into a number of tubes with and without added histidine and with and without asparagine.

	WITHOUT ASPARAGINE		0.15 PERCENT TREATED* ASPARAGINE		0.15 PERCENT ASPARAGINE	
	WITH HISTIDINE	WITHOUT HISTIDINE	WITH HISTIDINE	WITHOUT HISTIDINE	WITH HISTIDINE	WITHOUT HISTIDINE
Number of cultures	40	40	40	40	40	40
Number that failed to grow	28	39	27	40	27	29
Percent that failed to grow	70	97.5	67.5	100	67.5	72.5
Calculated average inoculum size	0.36	0.03	0.39	0	0.39	0.32

* The treated asparagine was passed through an adsorbent column of Filtrol-Neutrol which would be expected to remove the histidine.

In the first place when whole liquid cultures devoid of asparagine and histidine were plated out six hours after single h^- bacteria were introduced no increase in the number was found. Three out of ten plates contained single h^- colonies. This allows the calculation that the average inoculum size was 0.35 h^- bacteria. A separate determination of the average inoculum size by the direct plating of a 10^{-7} dilution of the inoculum yielded the value of 0.32 bacteria. Further, as table 8 indicates, when one ml of various dilutions of an h^- culture were introduced into liquid medium devoid of asparagine and histidine, growth occurred only to the extent predicted by separate platings of similar samples into agar devoid of asparagine and histidine.

TABLE 8

The number of h^+ organisms in an h^- culture determined by plating and by the inoculation of serial dilutions into medium devoid of added histidine and asparagine.

DILUTION	TOTAL NUMBER OF CULTURES IN LIQUID MEDIUM	NUMBER THAT FAILED TO GROW	PERCENT THAT FAILED TO GROW	CALCULATED NUMBER OF h^+	h^+ BY PLATE COUNT
10^{-1}	28	0	0	—	10
10^{-2}	40	15	38	0.97	1
10^{-3}	40	36	90	0.10	0
10^{-4}	40	40	100	0	0
10^{-5}	40	40	100	0	0
10^{-6}	40	40	100	0	0
10^{-7}	40	40	100	0	0

The formation of microcolonies by biochemical mutants of *Escherichia coli* has recently been observed by other investigators (WITKIN, private communication). This is in accord with our experience with mutants requiring other substances. Three other histidineless strains which formed microcolonies when plated onto agar with asparagine did not do so when the asparagine was omitted (table 9). Lysineless, methionineless and prolineless strains, even in the absence of asparagine, formed microcolonies which were subject to mutation on agar while an arginineless strain exhibited an increase with time in the number of reverted colonies although no microcolonies could be detected. A threonineless strain formed no reverted colonies and no microcolonies until a small amount of threonine (1γ per ml) was supplied to the agar. All of these strains were derived from the No. 15 strain of *Escherichia coli*. In addition, a derivative of the K-12 strain which was leucineless and threonineless (l^-t^-) exhibited the behavior shown in table 10. In the presence of threonine microcolonies were formed which mutated and gave rise to visible leucine-independent (l^+) colonies with time. In the presence of leucine, however, no microcolonies were detectable and, in this instance, the number of threonine-independent (t^+) colonies did not increase with time.

Inoculation of serial dilutions of a washed l^-t^- culture into liquid medium with threonine or with leucine gave results in accord with the behavior on agar. The growth that results in the presence of threonine after the inoculation of 10^{-2} to 10^{-6} dilutions is apparently due to the slow growth of the l^-t^- bacteria and the eventual back mutation of the l^- factor. Similarly, lysineless, arginineless, methionineless and prolineless strains, when inoculated into liquid medium, behave as though there were slow growth in the absence of these amino acids, with consequent mutation to independence. These experiments do not demonstrate that these mutants have only relative requirements and are capable of the slow synthesis of their respective amino acids. Although there was no asparagine present it may be that the glucose of the medium was contaminated with the very small amounts of amino acid necessary to bring about these phenomena. With our present knowledge it is impossible to be certain.

DISCUSSION

The experiments reported in this paper demonstrate that, when used with the proper precautions, the plating technique will allow an accurate and precise determination of the number of back-mutated[3] organisms in a culture of biochemical mutants of *Escherichia coli*. This allows a determination of the rate of mutation and a study of the mutation process itself. It has been, as a consequence, possible to show that the mutation of h^- to h^+ and l^- to l^+ is

[3] The term "back-mutated" is used in this instance in the absence of critical evidence that the factor(s) responsible for the biochemical deficiency have changed to a state where it enables growth factor synthesis. It has thus far been impossible to perform recombination experiments with strain No. 15 of *Escherichia coli* which would discriminate between back mutation and mutation in some other factor. All that is meant is that by mutation some bacteria have assumed the synthetic abilities of the original parent, that is, they have become prototrophic (RYAN and LEDERBERG 1946).

TABLE 9

The consequences of plating and inoculating serial dilutions of various biochemical mutants of Escherichia coli into minimal agar and liquid medium devoid of asparagine.

DILUTION	HISTIDINELESS			LYSINE-LESS	ARGININE-LESS	METHIO-NINE-LESS	THREO-NINE-LESS	PROLINE-LESS	TRYPTO-PHANE-LESS
	654-462	161-489	225-484	15L-171	1572 228	532-171	558-228	209-301	1250-228
				Growth in Liquid Minimal Medium					
10^{0}	+	+	+	+	+	+	−	+	+
10^{-1}	+	+	+	+	+	+	−	+	+
10^{-2}	+	−	−	+	+	+	−	+	−
10^{-3}	−	−	−	+	+	+	−	+	−
10^{-4}	−	−	−	+	−	+	−	+	−
10^{-5}	−	−	−	+	−	+	−	+	−
10^{-6}	−	−	−	−	−	+	−	+	−
10^{-7}	−	−	−	−	−	−	−	+	−
				Number of Colonies on Minimal Agar					
10^{0}	52	2	13	cloudy	45	30	0	many	19
10^{-1}	7	1	7	300+	102	9	0	400	17
10^{-2}	2	1	2	200+	36	0	0	233	0
10^{-3}	0	0	0	132+	5	157	0	33	1
10^{-4}	0	0	0	25	1	24	0	18	0
10^{-5}	0	0	0	5	1	16	0	4	0
10^{-6}	0	1	0	2	0	9	0	120	0
10^{-7}	0	0	0	0	0	11	0	53	0
T†	27	27	27	40	80	52	56	53	3

* Total number of bacteria ($\times 10^{7}$) per ml determined by layering with the required growth factor.

TABLE 10

The consequences of plating and inoculating serial dilutions of a leucineless-threonineless mutant (679-680) of Escherichia coli into minimal agar and liquid medium devoid of asparagine but in one instance containing threonine and in the other leucine.

DILUTION	PLUS 100γ DL-THREONINE PER ML					PLUS 100γ L-LEUCINE PER ML				
	TOTAL NUMBER OF CULTURES IN LIQUID MEDIUM	NUMBER THAT FAILED TO GROW	PERCENT THAT FAILED TO GROW	CALCULATED NUMBER OF REVERTED BACTERIA	NUMBER OF REVERTED COLONIES ON AGAR	TOTAL NUMBER OF CULTURES IN LIQUID MEDIUM	NUMBER THAT FAILED TO GROW	PERCENT THAT FAILED TO GROW	CALCULATED NUMBER OF REVERTED BACTERIA	NUMBER OF REVERTED COLONIES ON AGAR
10^{0}	10	0	0	—	32	10	0	0	—	1
10^{-1}	10	0	0	—	14	10	7	70	0.4	1
10^{-2}	10	1	10	2.3	1	10	10	100	0	0
10^{-3}	10	5	50	0.7	5	10	10	100	0	0
10^{-4}	10	5	50	0.7	1	10	10	100	0	0
10^{-5}	10	8	80	0.2	6	10	10	100	0	0
10^{-6}	10	9	90	0.1	—	10	10	100	0	—
10^{-7}	10	10	100	0	—	10	10	100	0	—
10^{-8}	10	10	100	0	—	10	10	100	0	—
10^{-9}	50	50	100	0	—	50	50	100	0	—
T†	—	—	—	69	—	—	—	—	69	—

† Total number of bacteria ($\times 10^{7}$) determined by inoculating one ml of the 10^{-9} dilution into 50 tubes containing both threonine and leucine.

spontaneous in the sense that it is not induced by the absence of histidine or of leucine from the testing plates (RYAN 1948). The rate of back-mutation of the h^- locus, calculated according to the formula of LURIA and DELBRÜCK (1943), is of the order of 5×10^{-8} per bacterium per generation.

It has not yet been discovered what role the formation of microcolonies may have played in the pregnant experiments of LEDERBERG (1947) on genetic recombination in biochemical mutants of *Escherichia coli*. We have used some of the same mutants (679-680, and also Y53) as he, and have shown that microcolonies form in the absence of leucine. In addition LEDERBERG used asparagine and, on occasion, unwashed agar which we have been able to show contains at least enough histidine to allow, in the absence of asparagine, microcolony formation by h^- bacteria. It must be stated that the plating of undiluted cultures, which LEDERBERG performed, provides the best condition for determining the number of prototrophs if the agar is contaminated with growth factor. On the other hand, since his experiments involve the mixture of two strains each possessing multiple nutritional requirements the growth of microcolonies may result in unanticipated events on the agar plate. For example, although "coincidental reversion at two or more loci is theoretically improbable and experimentally undemonstrable" when a strain is studied by itself, the role in mixtures of symbiotic microcolony enlargement with consequent mutation would be useful to know. It seems unlikely that LEDERBERG'S experiments have been qualitatively misinterpreted but the role of microcolonies certainly cannot be neglected.

The frequency with which microcolonies adapt to form visible reverted colonies was shown to be an inverse function of the total number per plate. This appears to be another example of the ability of biochemically deficient organisms to regulate the growth of more independent mutants. In the presence of leucine, leucineless mycelium of *Neurospora crassa* is able to prevent the overgrowth of mutant leucine-independent mycelium (RYAN 1946). This inhibition is a function of the proportion of the two types. Apparently, in some similar way, a large number of h^- bacteria, in the form of microcolonies, can decrease the chance that an h^+ mutant will escape and form an adapted macrocolony. This inhibition is even more strikingly demonstrated in experiments on growth in liquid medium which will be reported elsewhere (RYAN and SCHNEIDER 1948).

SUMMARY

Growth factor-independent mutants of *Escherichia coli* form visible colonies when biochemically deficient strains containing them are plated on minimal agar. Although otherwise reliable, this method of counting is confused by the disproportionately high number of mutant colonies formed on plates of members of low serial dilutions. This phenomenon was shown to be due to mutations occurring on the plates in microcolonies formed by the deficient bacteria in the absence of their required growth factors. This small amount of growth also occurs in liquid medium and allows mutations which result in

the eventual growth of biochemically deficient bacteria in the absence of their growth factors.

In the case of a histidineless strain it has been shown that the small amount of growth is due to traces of histidine in the medium. When these traces are removed the histidineless bacteria exhibit a complete requirement and do not divide at all in the absence of histidine. A culture of histidineless bacteria grows in liquid medium only when it contains some histidine-independent mutants. Similarly, plate counts of serial dilutions are normal.

A series of other biochemically deficient strains also form microcolonies in the absence of their required growth factors. In these cases it has not been possible to determine whether this is due to the slow synthesis of the required growth factors or whether these substances are present as minute traces in the minimal medium.

The behavior of microcolonies on minimal agar plates has been studied. They give rise in time to adapted macrocolonies consisting of growth factor-independent bacteria. The chance that one of these mutant bacteria will form an adapted colony is influenced by the deficient bacteria in the microcolony where it arises and by the total number of microcolonies on the plate. In this way biochemically deficient bacteria may regulate the adaptive expression of a back-mutation.

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